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APPLICATION NUMBER: *60/364,734*
FILING DATE: *March 14, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US02/30997*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL565098339US

J1060 U.S. PTO 03/14/02

J1040 U.S. PTO 60/364734 03/14/02

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Patrick D.	Kane	Anchorage, Alaska

☐ Additional inventors are being named on the _______ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

LOCALIZED NON-INVASIVE BIOLOGICAL MODULATION SYSTEM

CORRESPONDENCE ADDRESS

Direct all correspondence to:

[x] Customer Number: 26770 → Place Customer Number Bar Code Label here

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OR

[x] Firm or Individual Name	Ronald I. Eisenstein				
Address	Nixon Peabody LLP				
Address	101 Federal Street				
City	Boston	State	MA	ZIP	02110
Country	US	Telephone	617.345.6054	Fax	617.345.1300

ENCLOSED APPLICATION PARTS (check all that apply)

- [X] Specification — Number of Pages: 24
- [X] Drawing(s) — Number of Sheets: 27
- [ ] Application Data Sheet. See 37 CFR 1.76
- [ ] CD(s), Number: ____
- [X] Other (specify): See Provisional Application for Patent Cover Sheet (PTO/SB/16):

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

- [x] Applicant claims small entity status. See 37 CFR 1.27.
- [X] A check or money order is enclosed to cover the filing fees
- [ ] The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-0850
- [ ] Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

- [X] No.
- [ ] Yes, the name of the U.S. Government agency and the Government contract number are: ____________

Respectfully submitted,

SIGNATURE: [signature]

Date: 3/14/02

TYPED or PRINTED NAME: Ronald I. Eisenstein

REGISTRATION NO. (if appropriate): 30,628

TELEPHONE: 617.345.6054

Docket Number: 52250-P

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Addendum

Provisional Application for Patent Cover Sheet (PTO/SB/16): Additional Application Parts
Recordation Form Cover Sheet (1pg); Executed Assignment (2pp); 1 pg Abstract

PTO/SB/17 (11-01)
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FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 120.00

Complete If Known	
Application Number	To be assigned
Filing Date	Herewith
First Named Inventor	Patrick D. Kane
Examiner Name	Unknown
Group Art Unit	Unknown
Attorney Docket No.	52550-P

METHOD OF PAYMENT *(check all that apply)*

☒ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account: x Charge any deficiencies

Deposit Account Number: 50-0850

Deposit Account Name: Nixon Peabody LLP

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☐ Charge fee(s) indicated below ☒ Credit any overpayments

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	80

SUBTOTAL (1) ($) 80

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		X	=
Independent Claims		-3** =		X	=
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	40
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 40

SUBMITTED BY

				Complete *(if applicable)*	
Name *(Print/Type)*	Ronald I. Eisenstein	Registration No. *(Attorney/Agent)*	30,628	Telephone	617.345.6054
Signature	Ronald I. Eisenstein			Date	3/14/02

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PTO/SB/21 (08-00)
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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	To be assigned
Filing Date	Herewith
First Named Inventor	Patrick D. Kane
Group Art Unit	Unassigned
Examiner Name	Unassigned
Total Number of Pages in This Submission	Attorney Docket Number: 52550-P

ENCLOSURES *(check all that apply)*

- [X] Fee Transmittal Form
- [X] Fee Attached
- [ ] Amendment / Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [x] Assignment Papers *(for an Application)* 3 pages
- [X] Drawing(s) 27 sheets
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- [ ] Proprietary Information
- [ ] Status Letter
- [X] Other Enclosure(s) *(please identify below)*: See 1 in Addendum

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Ronald I. Eisenstein, Reg. No. 30,628
Signature	Ronald I. Eisenstein
Date	3/14/02

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231 on this date: [ ]

Typed or printed name	Patricia W. Turner		
Signature	Patricia W. Turner	Date	3/14/02

Addendum

1. Provisional Application Cover Sheet (2 pp)
Specification (24 pp); Abstract (1 pp); Fee Transmittal for FY 2002 (1 pg); Check for $120.00

Practitioner's Docket No. 52550-P

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Patrick D. Kane
Application No.: To be assigned
Filed: Herewith
For: LOCALIZED NON-INVASIVE BIOLOGICAL MODULATION SYSTEM

Group No.: Unknown
Examiner: Unknown

Assistant Commissioner for Patents
Washington, D.C. 20231

EXPRESS MAIL CERTIFICATE

"Express Mail" label number EL565098339US
Date of Deposit: March 14, 2002

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Transmittal Form (PTO/SB/21) (2 pp)
Fee Transmittal for FY 2002 (PTO/SB/17) (1 pg)
Provisional Application for Patent Cover Sheet (2 pp)
Specification (24 pp)
Drawings (27 sheets)
Abstract (1 pg)
Recordation Form Cover Sheet (1 pg)
Executed Assignment (2 pp)
Check for $120.00
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Patricia W. Turner

Patricia W. Turner

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Docket No. 52250-P
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LOCALIZED NON-INVASIVE BIOLOGICAL MODULATION SYSTEM

FIELD OF THE INVENTION

[001] The present application is directed to methods for localized non-invasive delivery of biological modulating agents throughout the body, particularly for the delivery of neuromodulators to specific sites within the brain.

BACKGROUND OF THE INVENTION

[002] There are a range of methods of delivery of an agent to a subject. For *in vivo* administration, methods include catheters, injection, scarification, etc. For example, stereotaxic injection can be used to direct delivery of an agent to a desired location in the brain. Stereotaxic surgery is performed using standard neurosurgical procedures [Pellegrino and Clapp, *Physiol. Behav.* 7: 863-8 (1971)]. Additionally, agents can be delivered by intracerebroventricular ("icv") infusion using a minipump infusion system, such as a SynchroMed Infusion System. A recent method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the viral particle to the target cell [R. Bobo et al., *Proc. Natl. Acad. Sci. USA* 91: 2076-80 (1994); P. Morrison et al., *Am. J. Physiol.* 266: R292-305 (1994)]. Other methods can be used including catheters, intravenous,

parenteral, intraperitoneal and subcutaneous injection, oral or other known routes of administration.

[003] However, many of these methods are systemic, or at best regional in application. This can result in delivery of an agent to normal tissues, where the effect of the agent can be deleterious. Thus, a method for targeted delivery of an agent to only a particular region would be desirable. It would also be desirable to do this in as non-invasive a manner as possible. Accordingly, localized targeted drug delivery is highly desirable for a wide array of applications. For example, the function of the central nervous system relies on the interconnectivity of specific subsets of neurons, which communicate using many different neurotransmitters. Many neurodegenerative diseases are characterized by loss of function of these connections, known as synapses. For example, Parkinson's Disease is a loss of dopaminergic activity in the pigmented neurons of the substantia nigra. Thus, it would be highly desirable to be able to deliver agents including drugs, genes, etc. in a non-invasive manner to a very specific site.

[004] The brain presents particular needs and challenges for targeted drug delivery.

[005] For example, the ability to excite or inhibit the activity of specific subsets of neurons in specific regions of the brain. The inability of many agents to cross the blood-brain barrier also causes problems.

[006] While sophisticated techniques for drug delivery have been developed, there remains a need for improved methods for the precise localized deposition of biologically active agents. Many existing methods rely on invasive techniques, such as localized injection to deliver an agent to its site of action. Even then the agent may disperse from that site. Moreover, such techniques are inherently fraught with the risks of infection

associated with any invasive procedure. Furthermore, certain tissues, such as the brain, are particularly sensitive to any intervention. Thus, it would be highly desirable to have a non-invasive method for the localized delivery of agents.

[007] One advance on drug delivery has been the development of liposomes, including time-release liposomes.

[008] Liposomes consist of at least one lipid bilayer membrane enclosing an aqueous internal compartment. Conventional liposomes are formulated to carry therapeutic agents, drugs or other active agents either contained within the aqueous interior space (water soluble active agents) or partitioned into the lipid bilayer (water-insoluble active agents). Active agents that have short half-lives in the bloodstream are particularly suited to delivery via liposomes. Many anti-neoplastic agents, for example, are known to have a short half-life in the bloodstream such that their parenteral use is not feasible. However, the use of liposomes for site-specific delivery of active agents via the bloodstream is limited by the rapid clearance of liposomes from the blood by cells of reticuloendothelial system (RES).

[009] Liposomes are normally not leaky but will become so if a hole occurs in the liposome membrane, if the membrane degrades or dissolves, or if the membrane temperature is increased to the phase transition temperature. The elevation of temperature (hyperthermia) at a target site in a subject to raise liposome temperature above the phase transition temperature, and thereby cause the release of the liposome contents, has been used for the selective delivery of therapeutic agents. Yatvin et al., Science 204:188 (1979). Recently liposome formulations capable of delivering

therapeutic amounts of active agents in response to mild hyperthermic conditions (U.S. Patent No. 6,200,598).

[0010] Thermosensitive liposomes have been developed which retain their structure at 37°C, human body temperature, but are destroyed at even slightly elevated temperatures (e.g. 42°C). Microwaves have been used for localized drug delivery by spatial localized destruction of thermosensitive liposomes (for example to treat tumors in the hand). However, microwaves do not offer a high degree of localization. Thus, in situations where precise control is desired, for example when targeting specific regions of the brain, it is not satisfactory. Thermosensitive liposomes have also been used with an invasive source of heat for localized drug delivery. However, as described above, such invasive techniques are associated with infection risks and are not available for all regions of the body.

[0011] Thus, there remains a need for improved methods of localized drug delivery. In particular, it would be highly desirable to have a non-invasive method for the localized delivery of biologically active molecules.

SUMMARY OF THE INVENTION

[0012] The present invention provides methods for non-invasive localized delivery of biologically active molecules, comprising packaging a molecule(s) of interest inside an energy sensitive vehicle, such as a thermosensitive vesicle, administering said vesicles to a subject, and inducing localized release of said molecules from said vesicles using a focused energy source. The thermosensitive vesicles include thermosensitive polymer

nanoparticles and thermosensitive liposomes. The vesicles may be delivered to a subject by any technique, including infusion. The molecules may be released from the vesicles using any non-invasive method which induces localized hypothermia, including focused ultrasound.

[0013] One preferred embodiment of the present invention provides methods for treating neural conditions, using any method that allows the vesicles to cross the blood-brain barrier to deliver neuromodulators to the brain. A preferred method for delivery to the brain is coating the vesicles with Polysorbate 80/85 or antibodies which are specifically targeted to the brain. Neuromodulators include molecules which activate or inhibit specific populations of neurons. Preferred neural conditions include epilepsy, Alzheimer's disease, Parkinson's disease, stroke, developmental learning disabilities, and post-traumatic neuronal cell loss.

[0014] Other preferred embodiments of the present invention provides methods for treating arthritis.

[0015] Another embodiment of the present invention provides a method for targeted adipose tissue destruction.

[0016] One preferred embodiment of the present invention provides a method for targeted gene therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figure 1 is a depiction of the formation of liposomes by the dispersion of lipid molecules in water, including the entrapment of drugs, antibodies, proteins, and peptides.

[0018] Figure 2 is a detailed depiction of a liposome.

[0019] Figure 3 depicts a standard procedure for preparing liposomes containing drugs (in either the lipid bilayer or inside the vesicle).

[0020] Figure 4 depicts Focused Ultrasound (FUS) methods, including their application to a subject's head using phased-array transducers.

[0021] Figure 5 depicts the use of magnetoliposomes for vesicle localization.

[0022] Figure 6 depicts the release of a neurotransmitter during the destruction of a liposome (for example, the destruction of a thermosensitive liposome in the presence of increased temperature).

[0023] Figure 7 depicts non-invasive neuronal modulation.

[0024] Figure 10A depicts the basic configuration of a nanovesicle, consisting of a aqueous core containing the desired agent (such as a neurotransmitter or drug) encapsulated by a membrane-block copolymer engineered to cause controlled, localized content release at a desired temperature, as depicted in Figure 10B, with the entire nanovesicle coated with polysorbate 80/85 to enhance transport efficacy across the blood brain barrier (BBB).

[0025] Figure 11 depicts an alternative configuration for the nanovesicle depicted in Figure 10, to which a targeting conjugate or coating has been added.

[0026] Figure 12 depicts another alternative nanovesicle configuration, in which subvesicle or subparticle is encapsulated in a nanovesicle.

[0027] Figure 8A depicts another alternative nanoparticle configuration, in which the desired agent is embedded directly in a polymer engineered to cause content release at a desired temperature, as shown in Figure 8B.

[0028] Figure 9 depicts the nanosphere of Figure 8, to which a conjugate or coating for targeting purposes has been added.

[0029] Figure 13A depicts a fusible liposome subvesicle encapsulated within the aqueous core of a nanovesicle. Figure 13B depicts the delivery of the liposome's contents to a cell via membrane fusion.

[0030] Figure 14 depicts another alternative nanovesicle configuration in which a desired agent is encapsulated in a polymer engineered for timed release and/or endocytic delivery within a nanovesicle engineered to cause content release at a desired temperature.

[0031] Figure 15 shows the results of release of GABA from lipid-polymer nanovesicles.

[0032] Figure 16 shows the results of release of GABA from lipid-polymer nanovesicles.

[0033] Figure 17 shows the results of release of GABA from lipid-polymer nanovesicles.

[0034] Figure 18 shows the results of release of GABA from lipid-polymer nanovesicles.

[0035] Figure 19 shows the results of release of GABA from lipid-polymer nanovesicles.

[0036] Figure 20 shows the results of release of GABA from lipid-polymer nanovesicles.

[0037] Figure 21 shows the results of release of GABA from lipid-polymer nanovesicles.

[0038] Figure 22 shows the results of release of GABA from lipid-polymer nanovesicles.

[0039] Figure 23 shows the results of release of GABA from lipid-polymer nanovesicles.

[0040] Figure 24 shows the results of release of GABA from lipid-polymer nanovesicles.

[0041] Figure 25 shows the results of release of GABA from lipid-polymer nanovesicles.

DETAILED DESCRIPTION OF THE INVENTION

[0042] We have now discovered a method for localized non-invasive delivery of biologically active molecules, comprising packaging a molecule of interest inside an energy-sensitive vesicle, preferably a thermosensitive vesicle, administering said vesicles to a subject, and inducing localized release of said molecules from said vesicles using a focused energy source.

Thermosensitive vesicles

[0043] Any thermosensitive vesicle which can package a molecule of interest and which is intact at body temperature (i.e. 37° C) but destroyed at any other, non-body temperature which can be tolerated by a subject may be used. Preferred thermosensitive vesicles include thermosensitive polymer nanoparticles and thermosensitive liposomes.

[0044] As used herein, nanoparticles include thermosensitive polymer nanovesicles, for example, including but not limited to, vesicles which are no smaller than 150 nanometers, and nanospheres, for example including but not limited to, spheres which are no smaller than 5 nanometers. Polymersome and nanovesicle are used interchangeably. Thermosensitive polymer nanovesicles are sometimes referred to as polymer nanovesicles or thermosensitive nanovesicles or simply nanovesicles.

[0045] A preferred vesicle is a polymeric nanoparticle, including nanospheres, nanovesicles, and polymersomes as depicted in Figure 10, consisting of a aqueous core containing the desired agent (such as a neurotransmitter or drug) encapsulated by a membrane-block copolymer engineered to cause content release at a desired temperature. Preferably, the entire nanovesicle is coated with polysorbate 80/85 to enhance transport efficacy across the blood brain barrier (BBB).

[0046] In another preferred embodiment, the nanoparticle may contain a targeting conjugate or coating, as depicted in Figure 11.

[0047] In another preferred embodiment, the nanoparticle may encapsulate a subvesicle or subparticle, as depicted in Figure 12.

[0048] In another preferred embodiment, the nanoparticle may contain the desired agent embedded directly in a polymer engineered to cause content release at a desired temperature, as depicted in Figures 8-9. Preferably this configuration can also include added conjugate or coatings for targeting purposes.

[0049] In another preferred embodiment, the nanoparticle may contain a fusible liposome subvesicle encapsulated within the aqueous core of a nanovesicle, as depicted in Figure 13A. Figure 13B depicts the delivery of the liposome's contents to a cell via membrane fusion.

[0050] In another preferred embodiment, the nanoparticle may consist of a desired agent encapsulated in a polymer engineered for timed release and/or endocytic delivery within a nanovesicle engineered to cause content release at a desired temperature, as depicted in Figure 14.

[0051] In another preferred embodiment, the thermosensitive vesicle is a thermosensitive liposome, sometimes referred to as a liposome.

[0052] Thermosensitive liposomes are known in the art. Liposomes according to the present invention may be prepared by any of a variety of techniques that are known in the art. See, e.g., U.S. Pat. No. 4,235,871; Published PCT applications WO 96/14057; New RRC, Liposomes: A practical approach, IRL Press, Oxford (1990), pages 33-104; Lasic D D, Liposomes from physics to applications, Elsevier Science Publishers, Amsterdam, 1993; Liposomes, Marcel Dekker, Inc., New York (1983). Entrapment of an active agent within liposomes of the present invention may also be carried out using any conventional method in the art. In preparing liposome compositions of the present invention, stabilizers such as antioxidants and other additives may be used as long as they do not

interfere with the purpose of the invention. Examples include co-polymers of N-isopropylacrylamide (*Bioconjug. Chem.* 10:412-8 (1999)).

[0053] A method of preparing a liposomal formulation according to the present invention comprises mixing the bilayer components in the appropriate proportions in a suitable organic solvent, as is known in the art. The solvent is then evaporated to form a dried lipid film. The film is rehydrated (at temperatures above the phase transition temperature of the lipid mixture) using an aqueous solution containing an equilibrating amount of the surface active agent and a desired active agent. The liposomes formed after rehydration can be extruded to form liposomes of a desired size, as is known in the art. For example, where liposomes composed of 80:20 DPPC:MPPC are produced, rehydration is carried out at a temperature above the phase transition temperature of this particular lipid mixture (above 39.degree.C.). The aqueous solution used to rehydrate the lipid film comprises an equilibrating amount of lysolipid monomers (e.g., a concentration equal to the Critical Micelle Concentration of MPPC).

[0054] Polyethylene glycol (PEG) may be incorporated into the liposome bilayer to inhibit fusion with undesired membranes (Bulte et al., *Proc. Intl. Soc. Mag. Reson. Med.*, Fifth Annual Meeting, p. 1596 (1997)).

[0055] The thermosensitive vesicle may include any other useful molecules. For example, the vesicle may include a monoclonal antibody on its surface which allows targeting of the vesicle to a desired site. For example, an antibody to the transferrin receptor, which can cross the blood-brain barrier, may be used to target vesicles to the brain. Similarly, membrane-colloidal magnetite (Fe304) may be incorporated into the liposome bilayer; the application of a magnetic field may then be used to localize the

vesicles to a desired site (Bulte et al., *Proc. Soc. Mag. Reson.*, Third Annual Meeting, p. 1139 (1995)).

[0056] The thermosensitive vesicles may be administered to a subject using known means. Oral administration and injection are preferred routes for administration.

Focused energy sources

[0057] Any focused energy source, preferably a heat source capable of inducing highly localized hyperthermia to promote the destruction of the thermosensitive vesicles may be used. For example, focused ultrasound.

Active Agents

[0058] As used herein, an active agent "in the interior" or "entrapped within" or "encapsulated in" the vesicle is that which is contained in the interior space of the vesicle, compared to that partitioned into the polymer membrane or lipid bilayer and contained within the vesicle membrane itself. As used herein, an active agent "within" or "entrapped within" or "encapsulated in" the polymer membrane of a nanoparticle or lipid bilayer of a liposome is carried as a part of the membrane, as opposed to being contained in the interior space of the nanoparticle or liposome.

[0059] Active agents may be in any form suitable for use in nanoparticles or liposomes, as is known in the art, including but not limited to aqueous solutions of active agents. Aqueous solutions of active agents within the nanoparticles or liposomes of the present invention may be at the same osmotic pressure as that of the body fluid of the intended subject, or at an increased osmotic pressure (see U.S. Pat. No. 5,094,854); the aqueous solutions may also contain some precipitated active agent, as is known in the art.

A preferred active agent for encapsulation in the interior of the nanoparticle or liposome is any water soluble, weak base agent.

[0060] The incorporation of certain active agents (such as some anesthetics) in nanoparticles or liposomes of the present invention may additionally alter (enhance or inhibit) the release of contents from the nanoparticle or liposome, or alter the transition temperature of the nanoparticle or liposome, compared to that which would be seen in a similar nanoparticle or liposome that did not contain the active agent.

[0061] The incorporation of certain active agents (such as some anesthetics) in nanoparticles or liposomes of the present invention may additionally alter (enhance or inhibit) the release of contents from the nanoparticle or liposome, or alter the transition temperature of the nanoparticle or liposome, compared to that which would be seen in a similar nanoparticle or liposome that did not contain the active agent.

[0062] The administration of antineoplastic or antitumor drugs such as doxorubicin, cisplatin and methotrexate using thermosensitive liposomes in combination with hyperthermia at the desired target site has been reported. See, e.g., Magin and Weinstein In: Liposome Technology, Vol. 3, (Gregoriadis, G., ed.) p. 137, CRC Press, Boca Raton, Fla. (1993); Gaber et al., Intl. J. Radiation Oncology, Biol. Physics, 36(5):1177 (1996).

[0063] Active agents suitable for use in the present invention include therapeutic drugs and pharmacologically active agents, nutritional molecules, cosmetic agents, diagnostic agents and contrast agents for imaging. As used herein, active agent includes pharmacologically acceptable salts of active agents. Suitable therapeutic agents include, for example, antineoplastics, antitumor agents, antibiotics, antifungals, anti-inflammatory agents, immunosuppressive agents, anti-infective agents, antivirals, anthelminthic, and

antiparasitic compounds. Methods of preparing lipophilic drug derivatives which are suitable for nanoparticle or liposome formulation are known in the art (see e.g., U.S. Pat. No. 5,534,499 to Ansell, describing covalent attachment of therapeutic agents to a fatty acid chain of a phospholipid).

[0064] Preferred active agents suitable for use in the present invention include neuromodulatory agents. Preferred neuromodulators include NMDA or AMPA receptor agonists, GABA agonists, and sodium or calcium channel blockers. Certain preferred neuromodulators are listed in Table 1.

TABLE 1: PREFERRED NEUROMODULATORS

STIMULANTS *(produce psychomotor arousal; treat attention deficit disorder)*		
Sympathomimetics	Dextroamphetamine Methylphenidate	Monoamine (DA & NE) agonist; increase release; block re-uptake
Cholinomimetics	Nicotine Muscarine	ACh agonist (high dose blocks)
Xanthines	Caffeine Theophylline	Block adenosine receptors; GABA antagonist
Convulsants	Strychnine	Glycine antagonist
DEPRESSANTS *(produce sedation; treat pain, anxiety, sleep disorders)*		
Opioids	Morphine, Codeine Heroin, Methadone	Endogenous opiate agonist
Barbiturates	Secobarbital	GABA agonist
Barbiturate-like	Meprobamate	Similar to barbiturates
Organic solvents	Alcohol Ether	Disrupt neuronal membrane; may facilitate GABA
HALLUCINOGENIC *(produce distorted perception)*		
NE-like	Mescaline	Alter 5HT activity
5HT-like	LSD	Alter 5HT activity
Other	Marijuana Anti-cholinergics, PCP	Alter 5HT activity

Drugs Used to Treat Psychological Disorders		
ANTIPSYCHOTICS *(treat schizophrenia; also delirium and dementia)*		
Phenothiazines	Chlorpromazine	Block DA receptors
Butyrophenones	Haloperidol	Block DA receptors
Other	Clozapine	Block DA receptors
ANTIDEPRESSANTS *(treat depression and bipolar disorder)*		
Tricyclics*(secondary amines)* Tricyclics (*tertiary amines*)	Nortriptyline Imipramine Clomipramine	Block NE re-uptake Block 5HT re-uptake Block 5HT re-uptake
Heterocyclics	Fluoxetine	Block NE & 5HT reuptake
MAO inhibitors	Phenelzine Tranylcypromine	Inhibit monoamine oxidase
Lithium	Lithium	Stabilizes synapses
ANTI-ANXIETY *(treat acute and chronic anxiety; also sleep disturbances)*		
Benzodiazepines	Alprazolam Diazepam	Faclitate GABA
Other	Buspirone	Decrease 5HT activity

Notes:

ACh = acetylcholine

DA = dopamine

5HT = serotonin

GABA = gamma amino butyric acid

LSD = lysergic acid diethylamide

MAO = monoamine oxidase

NE = norepinephrine

PCP = phencyclidine

[0065] Other preferred active agents include gene expression modulating agents, including activators such as tetracycline for use with Tet-activated promoters (for example, in transgenic animals).

[0066] Still another preferred embodiment of the present invention provides for a method of delivery of nucleic acids, such as cDNAs, in gene therapy treatments.

[0067] Another preferred class of active agents includes agents suitable for the treatment of stroke, including ischemic stroke. Examples of such preferred agents include thrombolytic agents such as tissue plasminogen activator or mannitol, or anticoagulants and antiplatelets such as warfarin, heparin, or aspirin. Such agents may be used in combination with other neuromodulators and/or neuroprotective agents.

[0068] In treating tumors or neoplastic growths, suitable compounds may include anthracycline antibiotics (such as doxorubicin, daunorubicin, carinomycin, N-acetyladriamycin, rubidazone, 5-imidodaunomycin, N30 acetyldaunomycin, and epirubicin) and plant alkaloids (such as vincristine, vinblastine, etoposide, ellipticine and camptothecin). Other suitable agents include paclitaxel (TAXOL.RTM.; a diterpenes isolated from the bark of the yew tree and representative of a new class of therapeutic agents having a taxane ring structure) and docetaxol (taxotere); mitotane, cisplatin, and phenesterine.

[0069] Anti-inflammatory therapeutic agents suitable for use in the present invention include steroids and non-steroidal anti-inflammatory compounds, such as prednisone, methyl-prednisolone, paramethazone, 11-fludrocortisol, triamciniolone, betamethasone and dexamethasone, ibuprofen, piroxicam, beclomethasone; methotrexate, azaribine, etretinate, anthralin, psoralins; salicylates such as aspirin; and immunosuppresant agents such as cyclosporine. Antiinflammatory corticosteroids and the antiinflammatory and immunosuppressive agent cyclosporine are both highly lipophilic and are suited for use in the present invention.

[0070] Additional pharmacologic agents suitable for use in nanoparticles of liposomes of the present invention include anesthetics (such as methoxyflurane,

isoflurane, enflurane, halothane, and benzocaine); antiulceratives(such as cimetidine); antiseizure medications such as barbituates; azothioprine (an immunosuppressant and antirheumatic agent); and muscle relaxants (such as dantrolene and diazepam).

[0071] Other preferred agents suitable for use in the present invention include molecules which promote bone healing. Such agents could be targeted for delivery to sites of bone fracture to reduce recovery time after an injury.

[0072] Imaging agents suitable for use in the present nanoparticle or liposome preparations include ultrasound contrast agents, radiocontrast agents (such as radioisotopes or compounds containing radioisotopes, including iodo-octanes, halocarbons, and renograf in), or magnetic contrast agents (such as paramagnetic compounds).

[0073] Nutritional agents suitable for incorporation into nanoparticles or liposomes of the present invention include flavoring compounds (e.g., citral, xylitol), amino acids, sugars, proteins, carbohydrates, vitamins and fat. Combinations of nutritional agents are also suitable.

Administration and Vesicle Size

[0074] Vesicles including polymer nanoparticles and liposomes of the present invention may be administered using methods that are known to those skilled in the art, including but not limited to oral administration, delivery into the bloodstream of a subject or subcutaneous administration of the vesicle. For example, the nanoparticles or liposomes may be administered by any suitable means that results in delivery of the nanoparticles or liposomes to the treatment site. It does not matter if the vesicle also goes to other sites because the agent will only be released where the energy source is directed.

For example, nanoparticles or liposomes may be administered intravenously and thereby brought to the treatment site by the normal blood flow; it is the precise heating of the targeted site that results in the vesicle membranes being heated to the phase transition temperature so that the vesicle contents are preferentially released only at the site of the tumor.

[0075] Where treatment of a tumor or neoplasm is desired, effective delivery of a vesicle-encapsulated active agent via the bloodstream requires that the nanoparticle or liposome be able to penetrate the continuous (but "leaky") endothelial layer and underlying basement membrane surrounding the vessels supplying blood to a tumor. Nanoparticles or liposomes of smaller sizes have been found to be more effective at extravasation into tumors through the endothelial cell barrier and underlying basement membrane which separates a capillary from tumor cells. See, e.g., U.S. Pat. No. 5,213,804 to Martin et al.

[0076] As used herein, "solid tumors" are those growing in an anatomical site other than the bloodstream (in contrast to blood-borne tumors such as leukemias) Solid tumors require the formation of small blood vessels and capillaries to nourish the growing tumor tissue.

[0077] It will further be appreciated that the vesicles of the present invention may be utilized to deliver of anti-infective agents to sites of infection, via the bloodstream. The use of for example, nanoparticles or liposomes containing a vesicle-forming lipid derivatized with a hydrophilic polymer, and having sizes ranging between 0.07 and 0.2 microns, to deliver therapeutic agents to sites of infection is described in published PCT patent application WO 93/19738. In accordance with the present invention, the anti-

infective agent of choice is entrapped within a nanoparticle or liposome having a membrane according to the present invention, and the resulting vesicle formulation is administered parenterally to a subject, preferably by intravenous administration. If desired, localized hyperthermia may be induced at the site of infection to cause the preferential release of liposomal contents at that site.

[0078] The size of vesicles in a preparation will depend upon the active agent contained therein and/or the intended target. Vesicles of between 0.05 to 0.3 microns in diameter are suitable for tumor administration (U.S. Pat. No. 5,527,528 to Allen et al.) Sizing of vesicles according to the present invention may be carried out according to methods known in the art, and taking into account the active agent contained therein and the effects desired (see, e.g., U.S. Pat. No. 5,225,212 to Martin et al; U.S. Pat. No. 5,527,528 to Allen et al). A preferred embodiment of the present invention is a vesicle of less than 10 microns in diameter, or a vesicle preparation containing a plurality e.g., liposomes of less than 10 microns in diameter. In a further preferred embodiment of the present invention, vesicles are from about 0.05 microns or about 0.1 microns in diameter, to about 0.3 microns or about 0.4 microns in diameter. Vesicle preparations may contain vesicles of different sizes.

[0079] In another preferred embodiment of the present invention, vesicles are from about 50 nm, 100 nm, 120 nm, 130 nm, 140 nm or 150 nm, up to about 175 nm, 180 nm, 200 nm, 250 nm, 300 nm, 350 nm, 400 nm or 500 nm in diameter.

[0080] In one aspect of the present invention, the vesicles are prepared to have substantially homogeneous sizes in a selected size range. One effective sizing method involves extruding an aqueous suspension of the vesicles through a series of

polycarbonate membranes having a selected uniform pore size; the pore size of the membrane will correspond roughly with the largest sizes of liposomes produced by extrusion through that membrane. See e.g., U.S. Pat. No. 4,737,323 (Apr. 12, 1988).

[0081] In a further aspect of the present invention, vesicles are dispersed in physiological saline or PBS to provide an aqueous preparation of vesicles. For example the aqueous preparation may further include an equilibrating amount of the surface active agent contained in the liposome bilayer, to reduce or prevent loss of the surface active agent from the liposome bilayer into solution. Liposomes composed of DPPC:MPPC may be contained in physiological saline or PBS that contains from about 1 mmM to about 5 mM of MPPC monomer.

[0082] The amount of active agent to be entrapped within or carried by liposomes according to the present invention will vary depending on the therapeutic dose and the unit dose of the active agent, as will be apparent to one skilled in the art. In general, however, the preparation of vesicles of the present invention is designed so the the largest amount of active agent possible is carried by the vesicle. Vesicles of the present invention may be of any type, however, LUVs are particularly preferred.

Applications

[0083] The method of the present invention may be used for the localized delivery of a wide variety of agents to treat a wide variety of conditions. For example, the delivery of neuromodulators to specific regions of the brain may be used to modulate neuronal transmission, including the delivery of inhibitory neurotransmitters to treat seizure foci in epileptics; excitatory neurotransmitters to treat Alzheimer's patients; excitatory neurotransmitters to enhance dopaminergic activity in Parkinson's patients; inhibitory

neurotransmitters (such as NMDA antogonists) to prevent brain damage in stroke victims, including emergency stroke treatment; agents to treat developmental learning disabilities (such as ADHD); and neurotransmitters to prevent post-traumatic neuronal cell loss. The method of the present invention may also be used to deliver anti-arthritic agents such as anti-inflammatory drugs to sites of athritic lesions in arthritis patients. Another embodiment prevents localized deposition of agents to treat artherosclerotic lesions. In another embodiment, the present method may be used to deliver cytotoxic agents for localized tissue destruction, including for example solid tumors as well as undesired adipose tissue. A further embodiment of the present invention provides the localized delivery of nucleic acids for targeted gene therapy.

EXAMPLES

Epilepsy (rodent)

Subject animal implanted with seizure inducing substance and recording device
Subject injected with vesicle packaged inhibitory neurotransmitter
Immediately following seizure instigation, stimulation electrodes deactivated
Seizure propagation monitored by surface EEG
tFUS activated and focused on seizure foci
Inhibitory neurotransmitter released at seizure foci and epileptiform activity subdued
Inhibitory neurotransmitter release is continued and slowly reduced, inducing LTD and extinguishing the chance of epileptic relapse

Alzheimer's (rodent)

Subject animal bred with Alzheimer's dementia mutation
Control, non-Alzheimer's, non-tFUS animal run through memory task
Alzheimer's animal run through an identical memory task, demonstrating diminished task completion ability
Subject injected with vesicle packaged excitatory neurotransmitter
tFUS targeted to hippocampal region of Alzheimer's subject
Synthetic θ–rhythm induced in hippocampal formation of Alzheimer's subject, replacing function of deteriorated septal cholinergic cells, and enhancing memory retention

Alzheimer's subject run through memory task under tFUS influence, demonstrating normal to exemplary task completion ability

Parkinson's (rodent)

Subject animal bred with Parkinson's mutation
Control, non-Parkinson's, non-tFUS animal run through a motor function related task
Parkinson's animal run through an identical memory task, demonstrating diminished task completion ability
Subject injected with vesicle packaged excitatory neurotransmitter
tFUS targeted to substantia nigra (pars compacta) of Parkinson's subject, enhancing dopaminergic activity, and demonstrating normal task completion ability
tFUS also focused on pathways utilized by aforementioned region to modulate other areas of the basal ganglia and premotor cortex, further alleviating the tremor and inability to initiate movement prevalent in Parkinson's patients

Stroke (rodent)

Local population of subject animal neurons damaged due to lack of blood flow to region.
Target release of inhibitory neurotransmitter surrounding damaged area prevents disabling brain damage.

Emergency stroke treatment:
Though initial damage caused by strokes is due mainly to lack of oxygen to cells, this usually only causes permanent damage to a small amount of cells. The main damage is due to the subsequent apoptosis and surrounding cell death caused by excessive depolarization and calcium influx. Depositing local modulators (e.g. NMDA antagonist) would prevent brain damage to critical brain regions while allowing the patient to sustain activity in neural systems controlling heart function, breathing, and the like while s/he heals.

DLD (rodent)

A.
Subject animal with DLD (i.e. ADHD) is monitored for hyperactivity during behavioral task
Control subject is injected with ADHD medication and monitored for side effects
Test subject injected with vesicle packaged ADHD medication
tFUS targeted to area relevant to ADHD cause
Medication released only in necessary areas, reducing and eliminating adverse side effects common to ADHD medication use
Subject run through behavioral task under and after UEDP treatment , demonstrating hyperactivity extinction

B.

Subject animal with DLD (i.e. ADHD) is monitored for hyperactivity during behavioral task
Control subject is injected with ADHD medication and monitored for side effects
Test subject injected with vesicle packaged neurotransmitter
tFUS targeted to areas relevant to ADHD cause
Excitability and activity modulated in relevant areas
Subject run through behavioral task under and after UEDP treatment , demonstrating hyperactivity extinction

Post-Traumatic Neuronal Cell Loss (rodent)

Subject animal with Neurotoxicity monitored during behavioral task
Subject injected with vesicle packaged neurotransmitter
tFUS targeted to damaged areas
Excitability and activity manipulated to reestablish normal plasticity in affected brain regions
Subject run through behavioral task, demonstrating reestablished brain function

hLTP (primate)

Subject run through memory task; completion capability recorded
Subject injected with vesicle packaged excitatory neurotransmitter
tFUS targeted to hippocampus
θ–rhythm oscillation modulated, reinforcing septal inputs and allowing control over stimuli retention
Subject run through memory task under tFUS influence, demonstrating increased stimuli retention, associational ability, and learning speed

hLTD (primate)

A.
Subject run through memory task; completion capability recorded
Subject injected with vesicle packaged inhibitory neurotransmitter
tFUS targeted to hippocampus
Subject run through different memory task (of same difficulty) under LTD inducing tFUS influence, demonstrating diminished stimuli retention, and temporary loss of learning ability

B.

Subject run through multi-trial memory task; increasing completion speed recorded
Subject injected with vesicle packaged inhibitory neurotransmitter
tFUS targeted to hippocampus; regional LTD induced
Subject run through same memory task; completion capability recorded, demonstrating loss of previously gained memory

NOTE: Though initial experimentation is confined to the hippocampus, plasticity modulation is not limited to any single brain region. In fact, expanding hLTP/hLTD manipulation to other regions will enhance desired effects, and is a natural next step.

Arthritis (rodent)

Subject animal with arthritis injected with vesicle packaged, arthritis-killing drug
tFUS targeted to arthritis location
High potency drug selectively released , demonstrating arthritis destruction with insignificant or zero damage to surrounding cells

NOTE: Though arthritis is destroyed in the above experiment, this procedure is not limited to a particular condition. Any spatially localized disease can be annihilated by this drug delivery method (e.g. cancer).

Targeted Fat Cell Destruction (rodent)

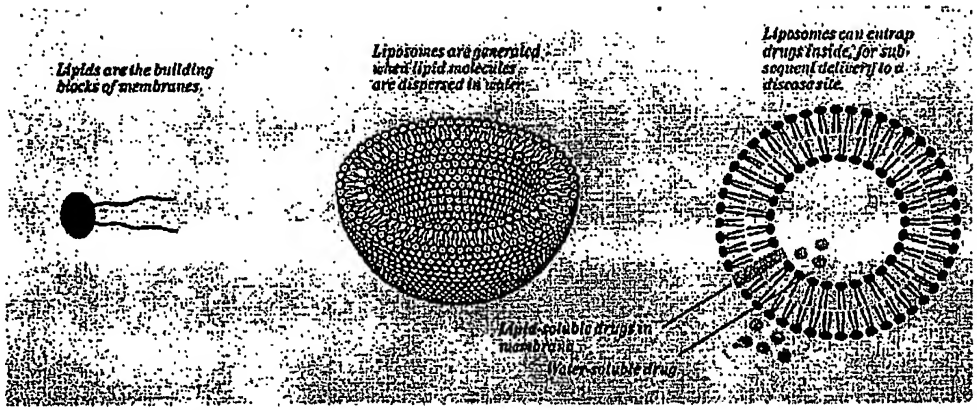
Overweight subject is injected with vesicle packaged, fat-cell-killing compound
tFUS is targeted to unwanted fat excess, demonstrating localized fat annihilation

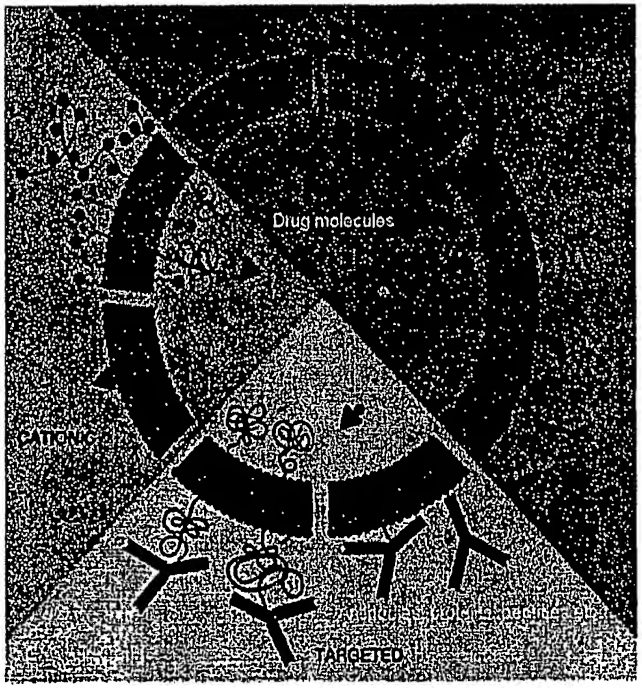
All references described herein are incorporated herein by reference.

ABSTRACT

The present invention provides methods for non-invasive localized delivery of biologically active molecules, comprising packaging a molecule(s) of interest inside a thermosensitive vesicle, administering said vesicles to a subject, and inducing localized release of said molecules from said vesicles using a focused heat source. The thermosensitive vesicles may be thermosensitive polymer nanoparticles or thermosensitive liposomes. The vesicles may be delivered to a subject by any technique, including infusion. The molecules may be released from the vesicles using any method which induces localized hypothermia, including focused ultrasound.

Liposome





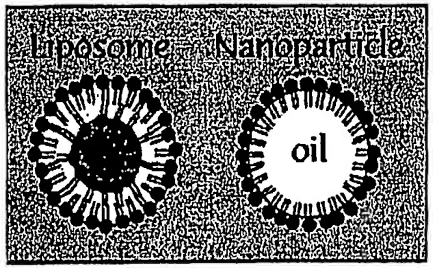


Fig. 1

Liposome Package

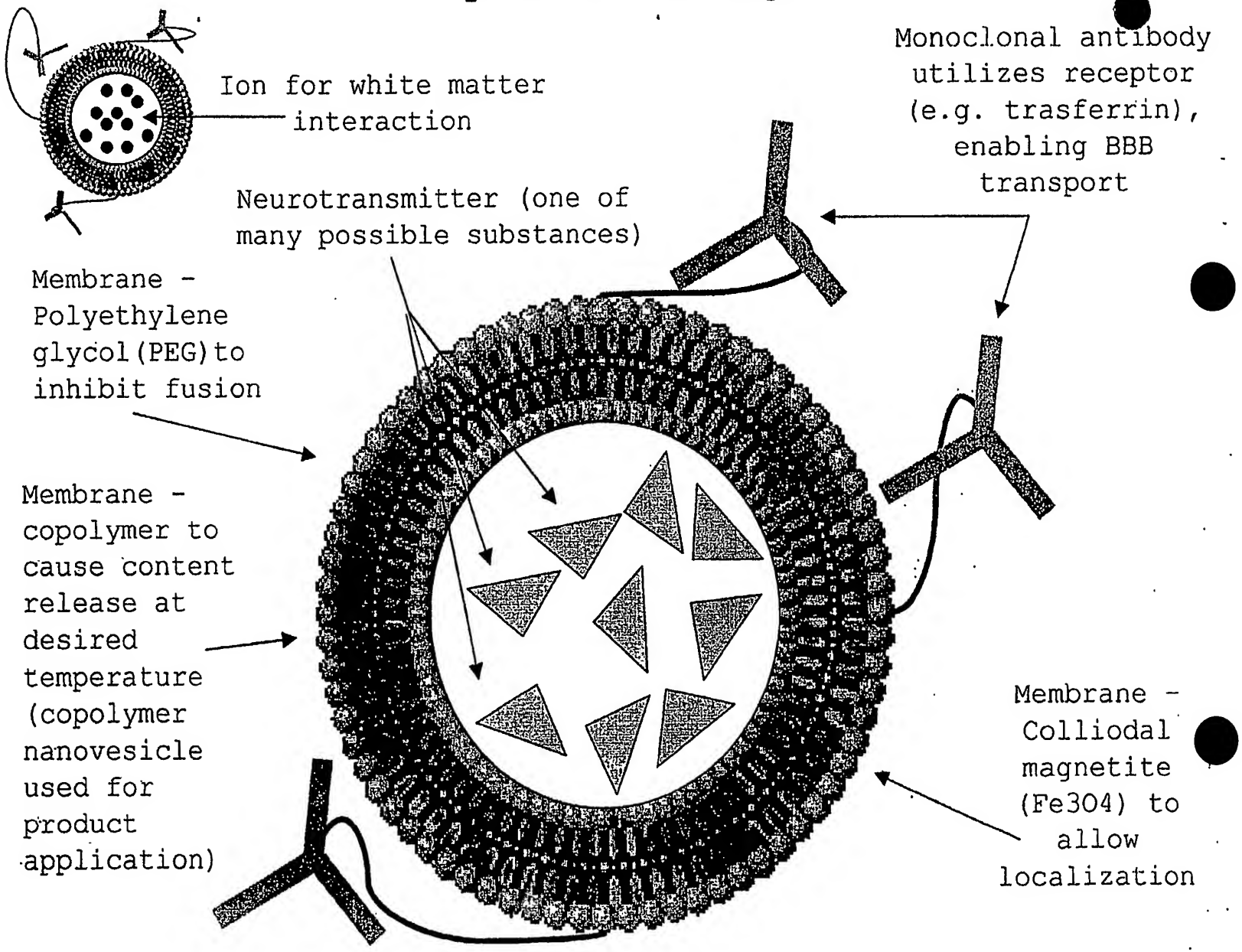


Fig. 2

Liposome Preparation

Standard Procedure

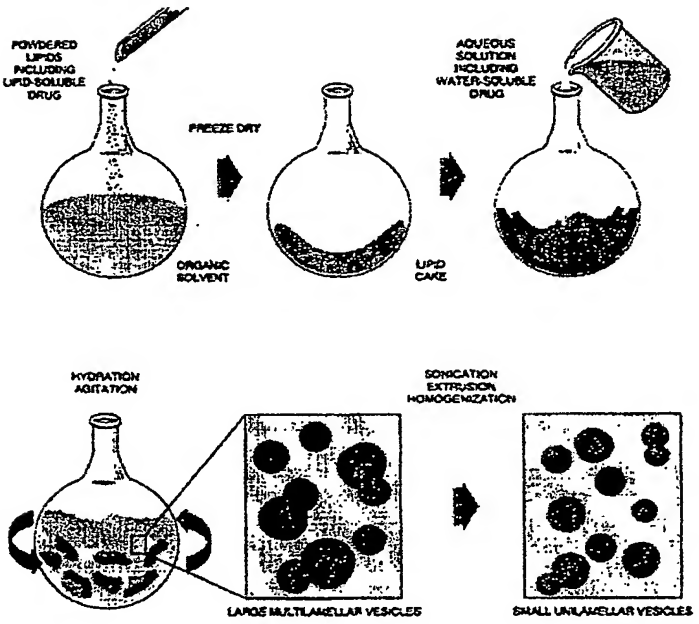
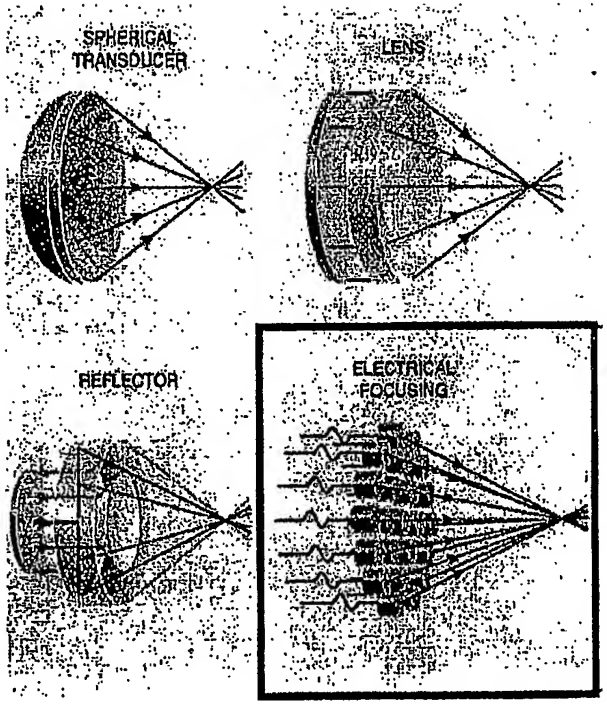


Fig. 3

Controlled Heat Deposition

Focused Ultrasound (FUS)
Methods



Phased-array
Transducers

Surface EEG

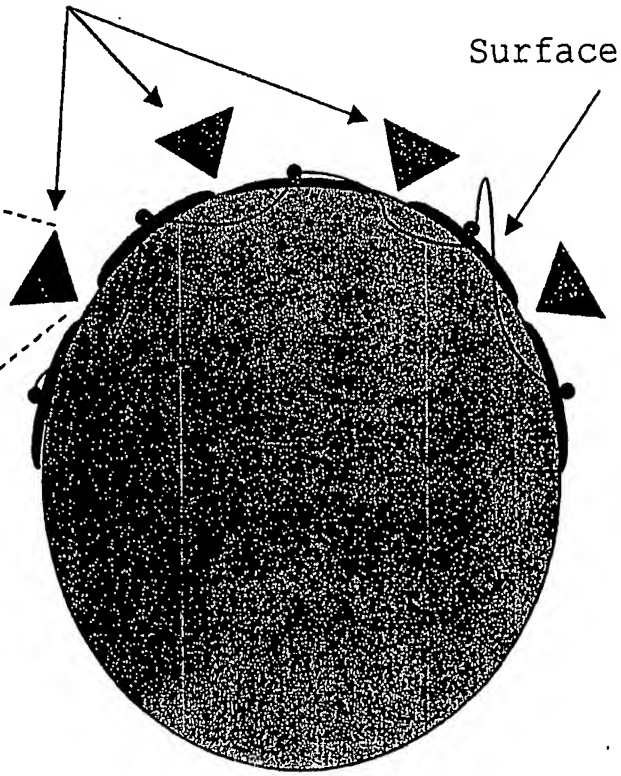


Fig. 4

Liposome Package Localization

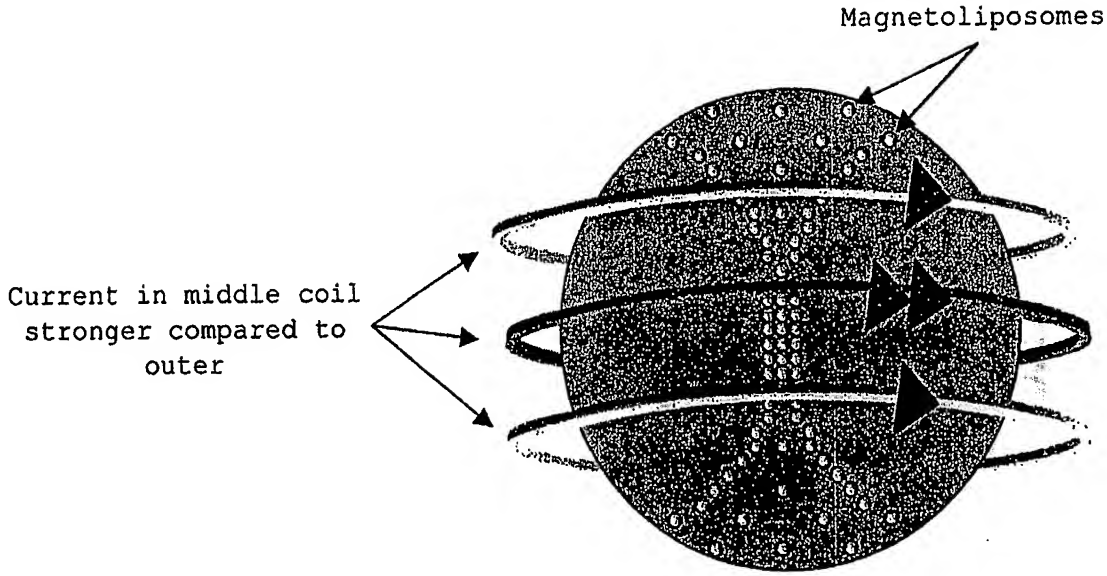


Fig. 5

Package Destruction

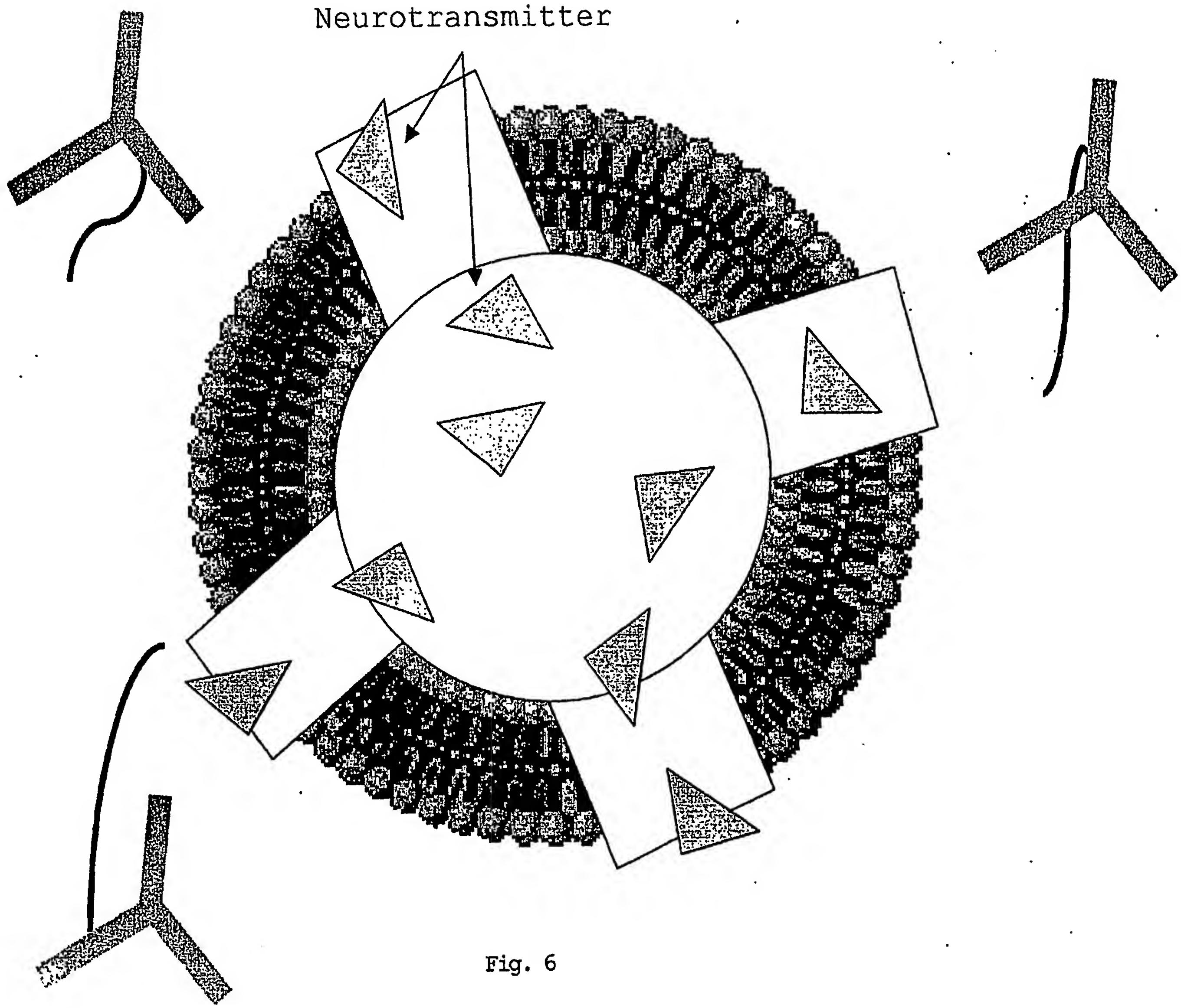


Fig. 6

Non-invasive Neuronal Modulation

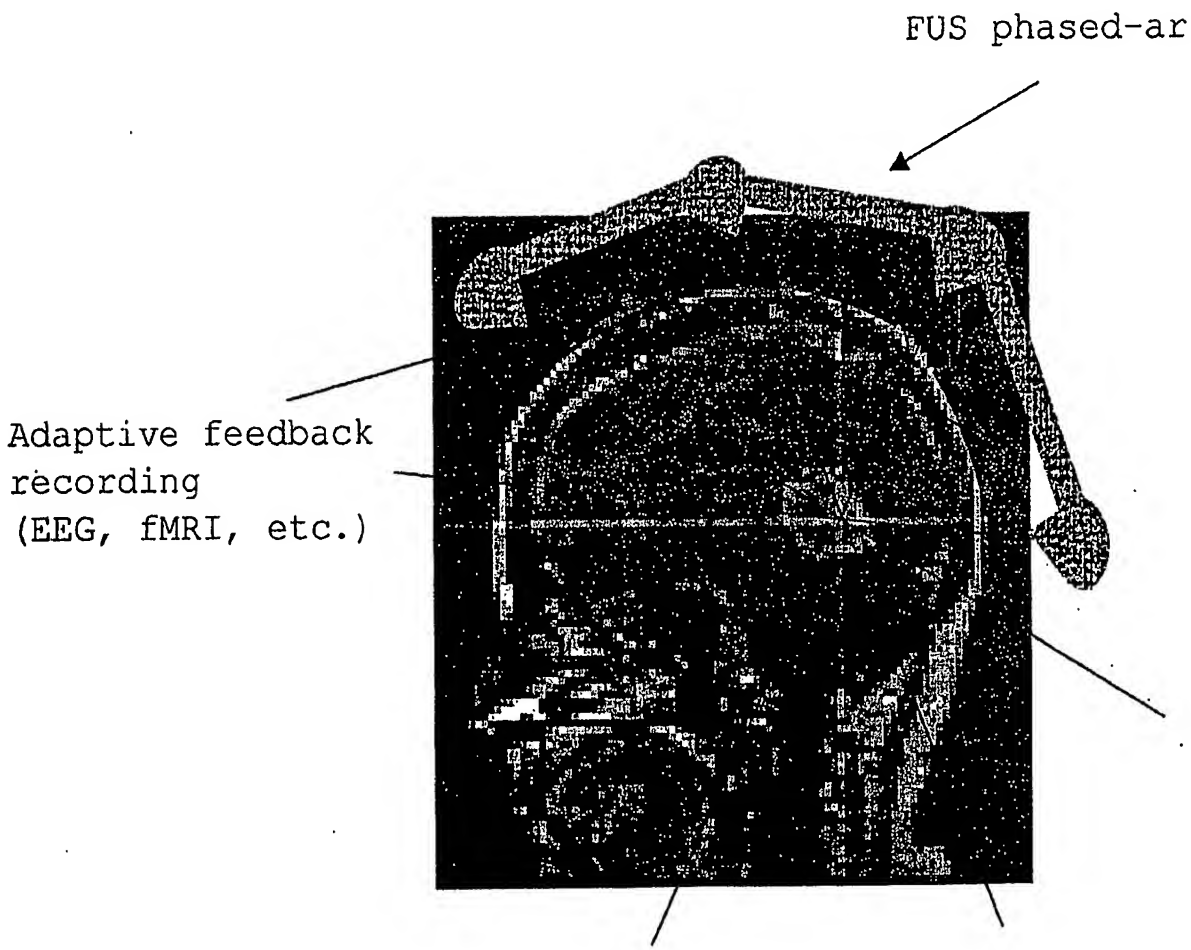


Fig. 7

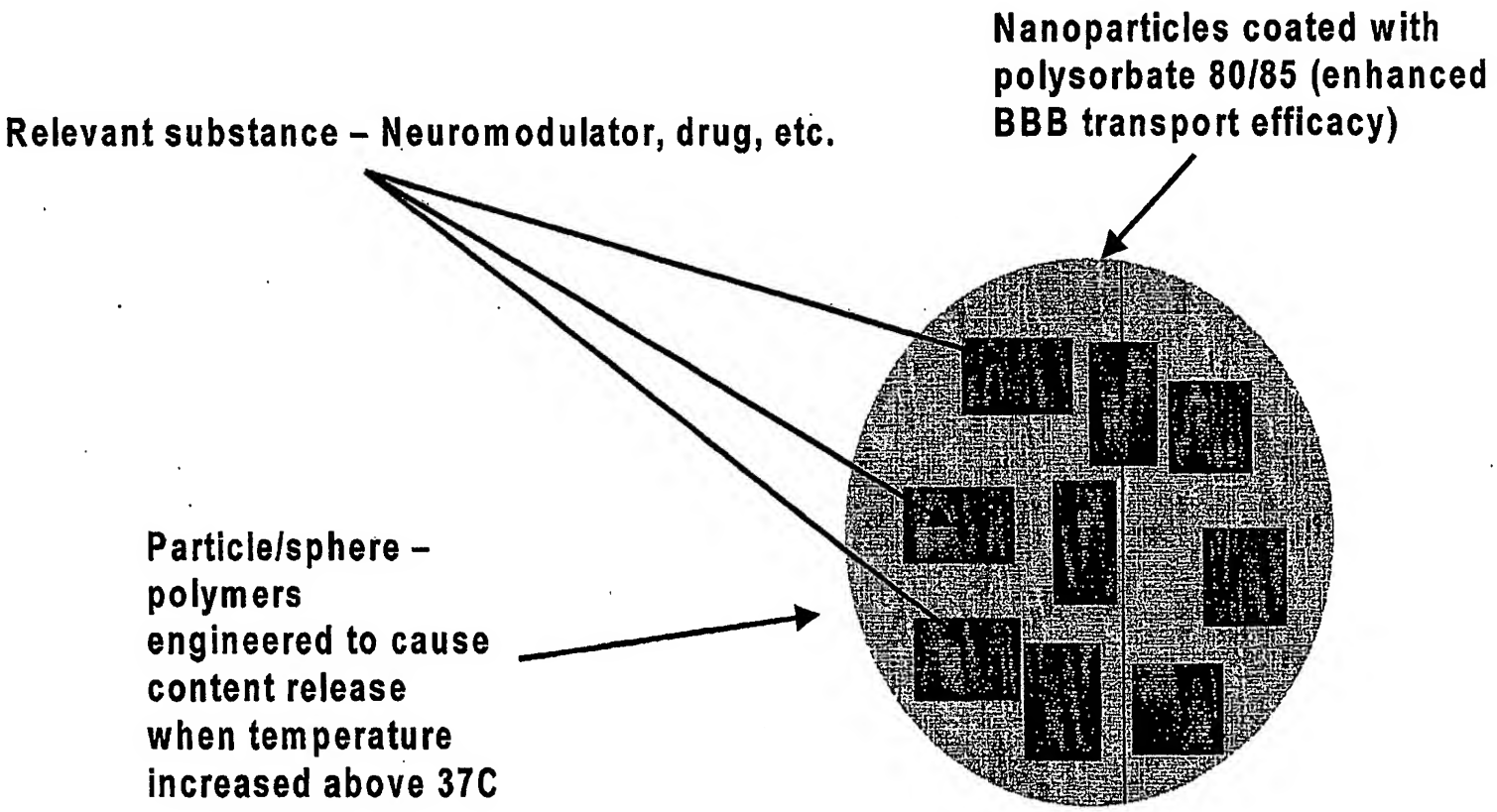

Nanoparticles coated with polysorbate 80/85 (enhanced BBB transport efficacy)
Relevant substance – Neuromodulator, drug, etc.
Particle/sphere – polymers engineered to cause content release when temperature increased above 37C

Fig. 8A

Spatially/temporally restricted
active agent release

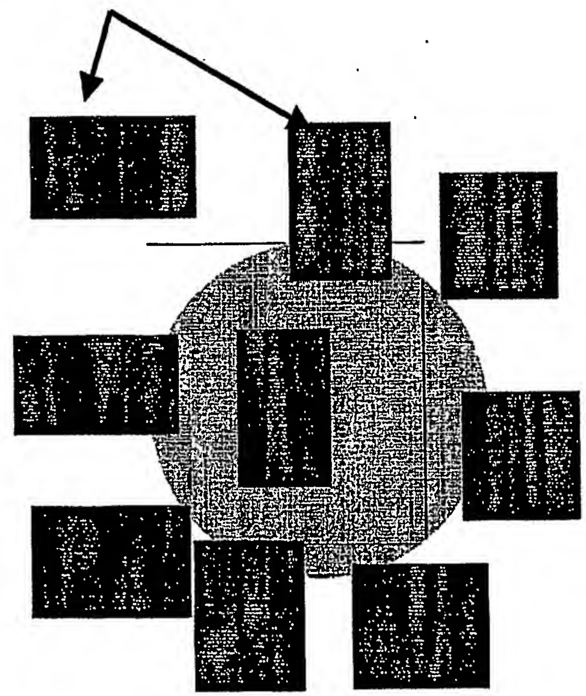


Fig. 8B

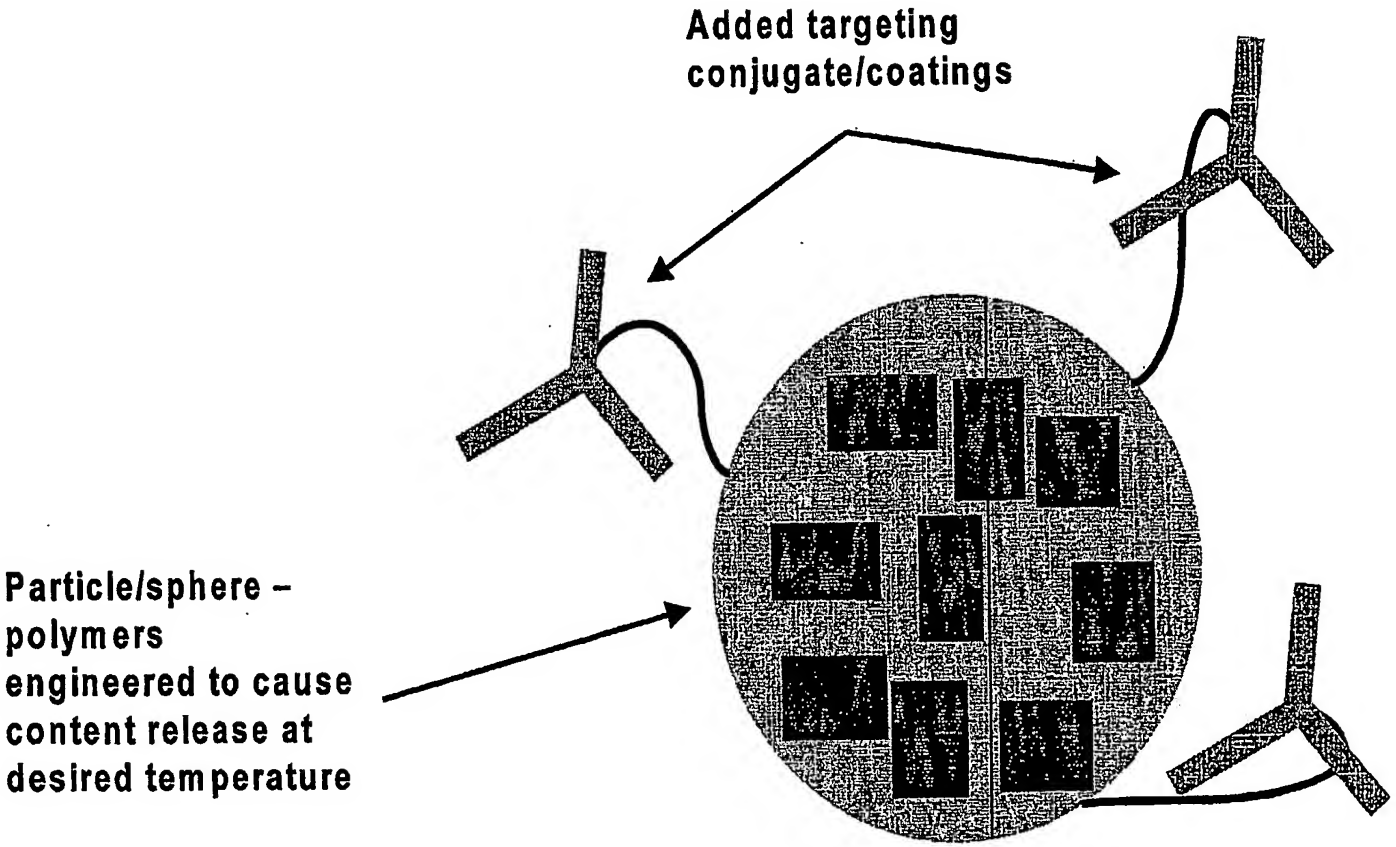


Fig. 9

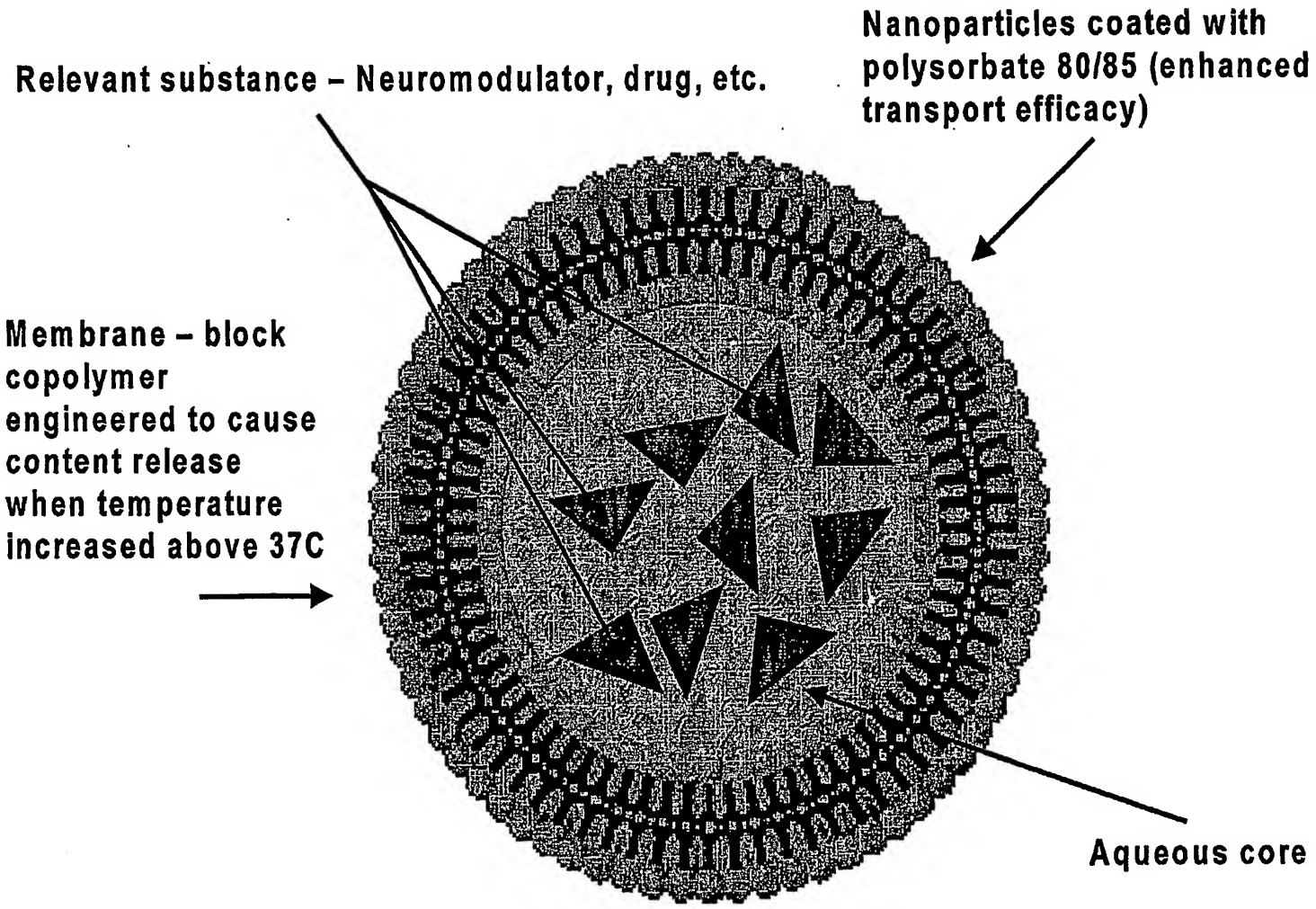

Relevant substance – Neuromodulator, drug, etc.
Nanoparticles coated with polysorbate 80/85 (enhanced BBB transport efficacy)
Membrane – block copolymer engineered to cause content release when temperature increased above 37C
Aqueous core

Fig. 10A

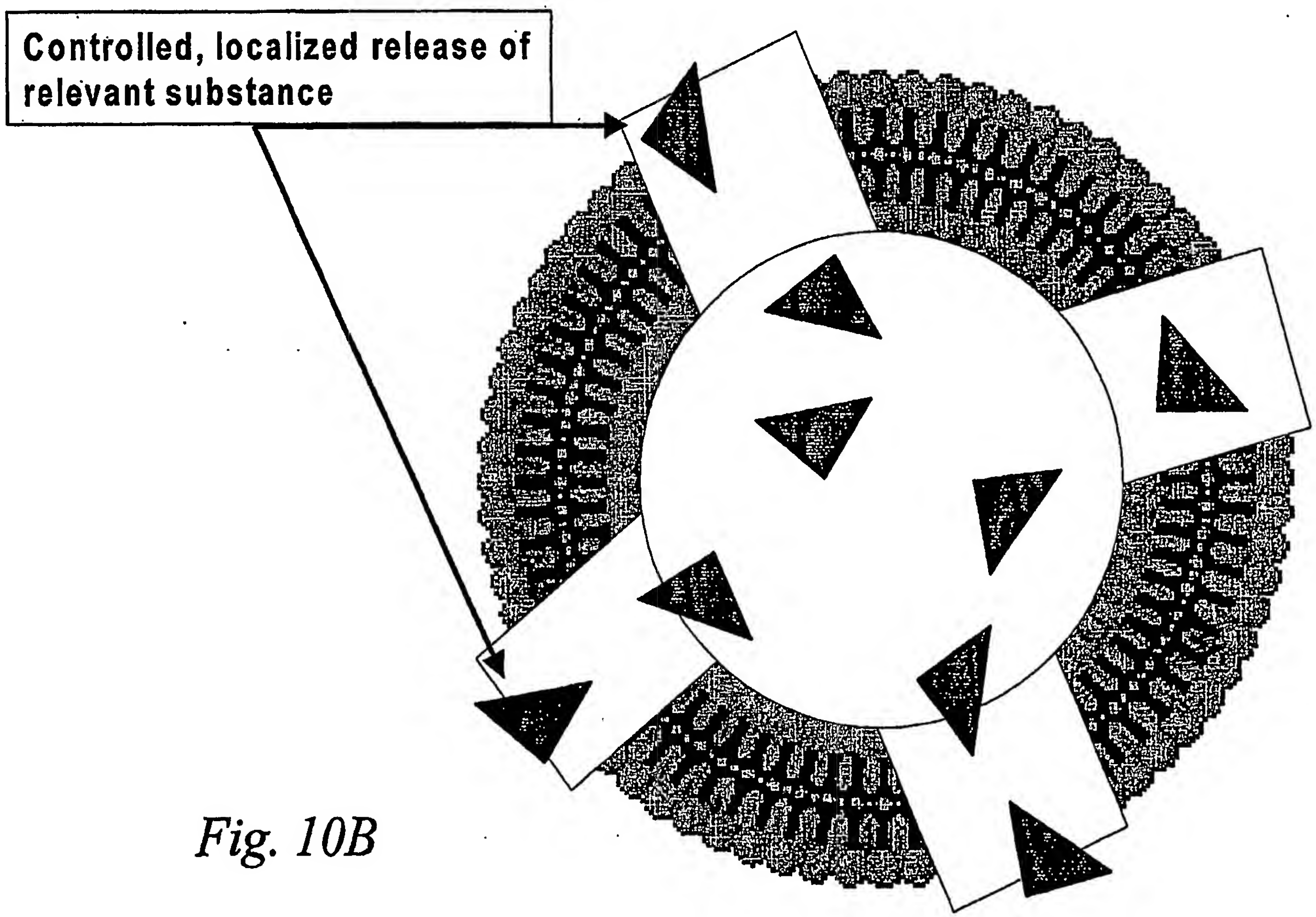


Fig. 10B

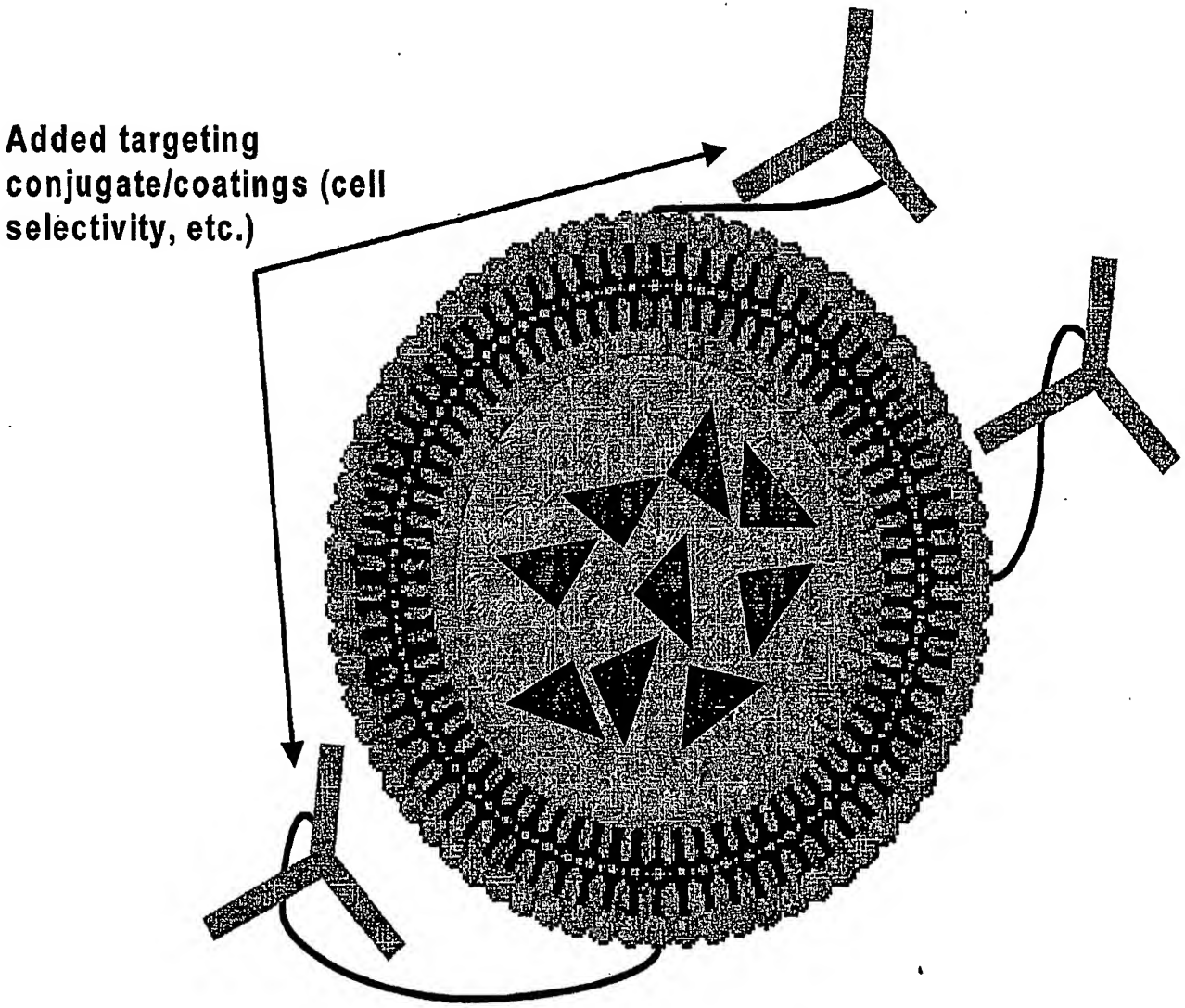


Fig. 11

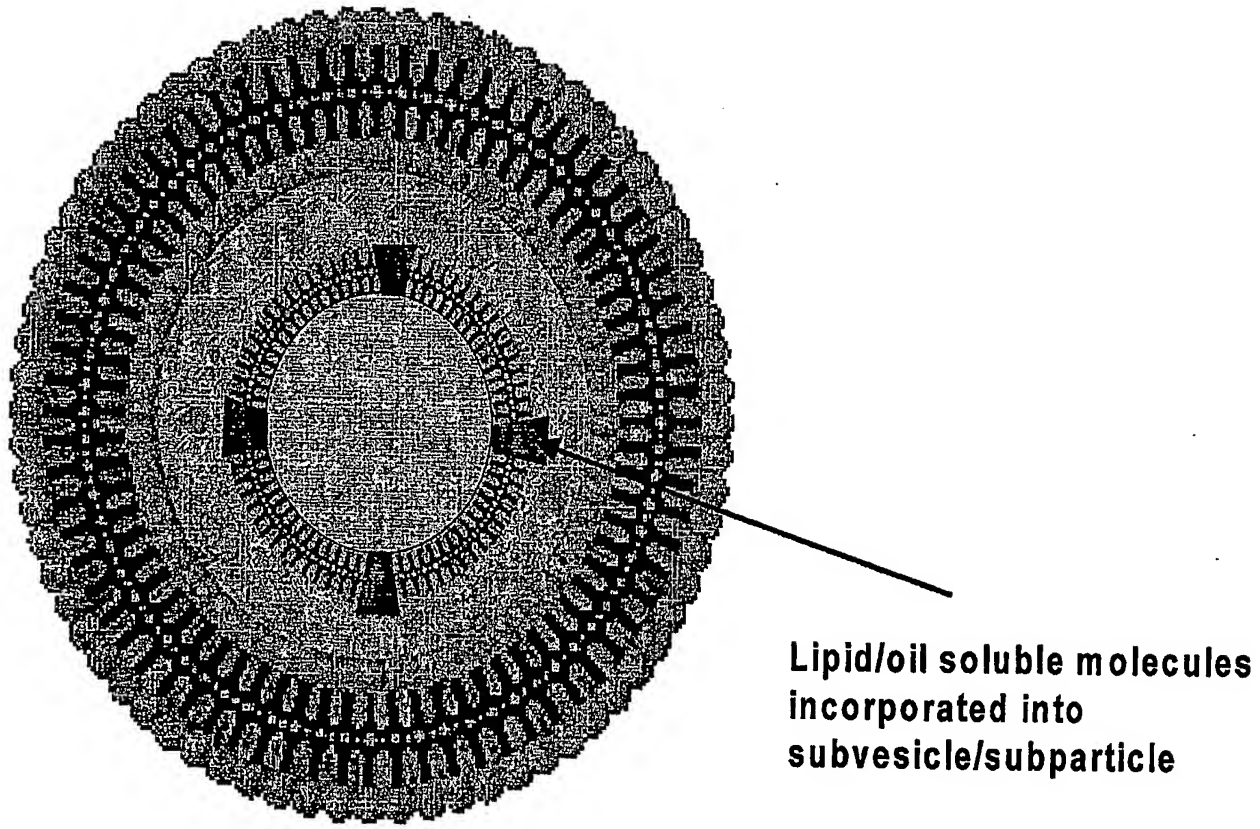


Fig. 12

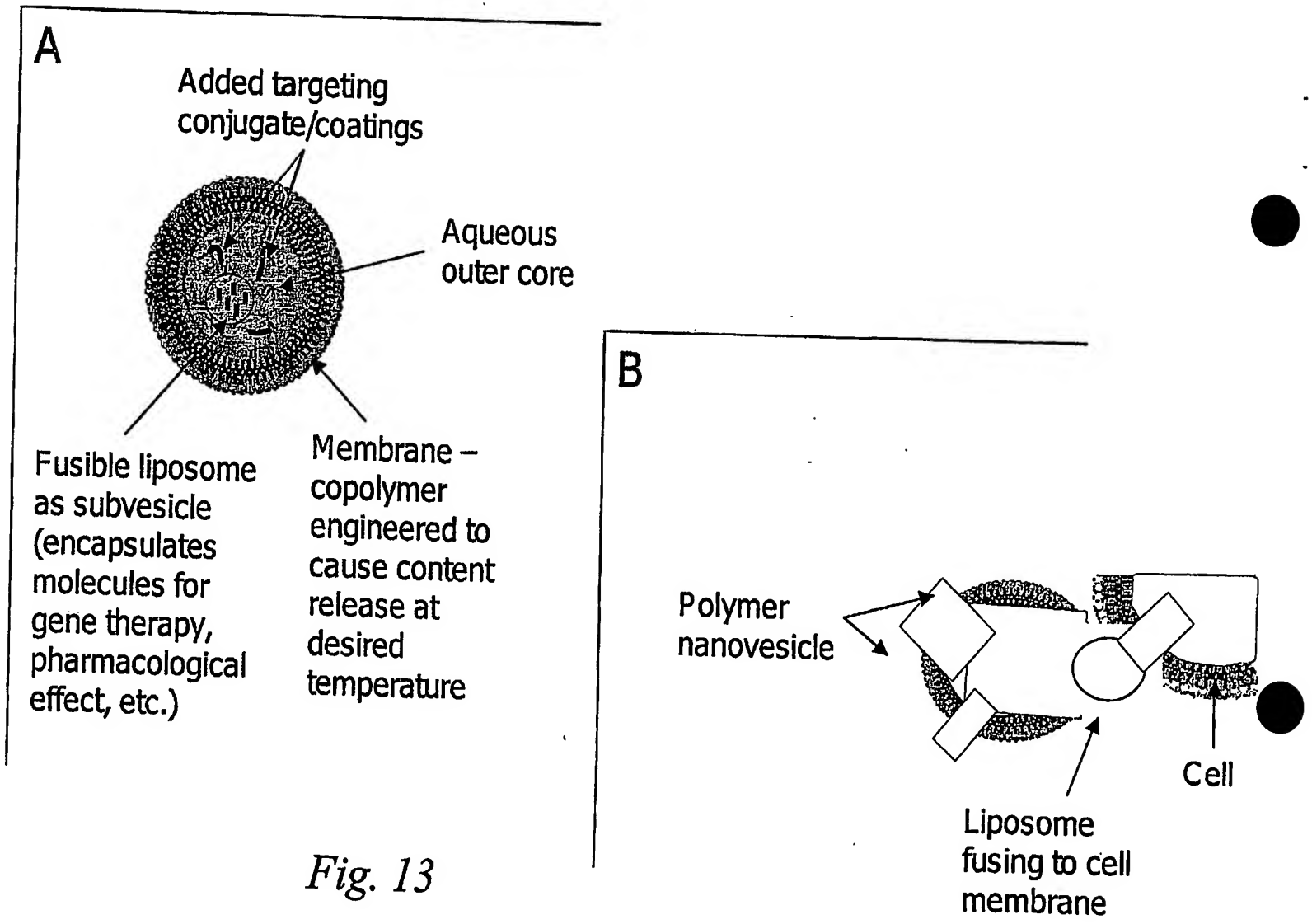

A
Added targeting conjugate/coatings
Aqueous outer core
Fusible liposome as subvesicle (encapsulates molecules for gene therapy, pharmacological effect, etc.)
Membrane – copolymer engineered to cause content release at desired temperature
B
Polymer nanovesicle
Cell
Liposome fusing to cell membrane

Fig. 13

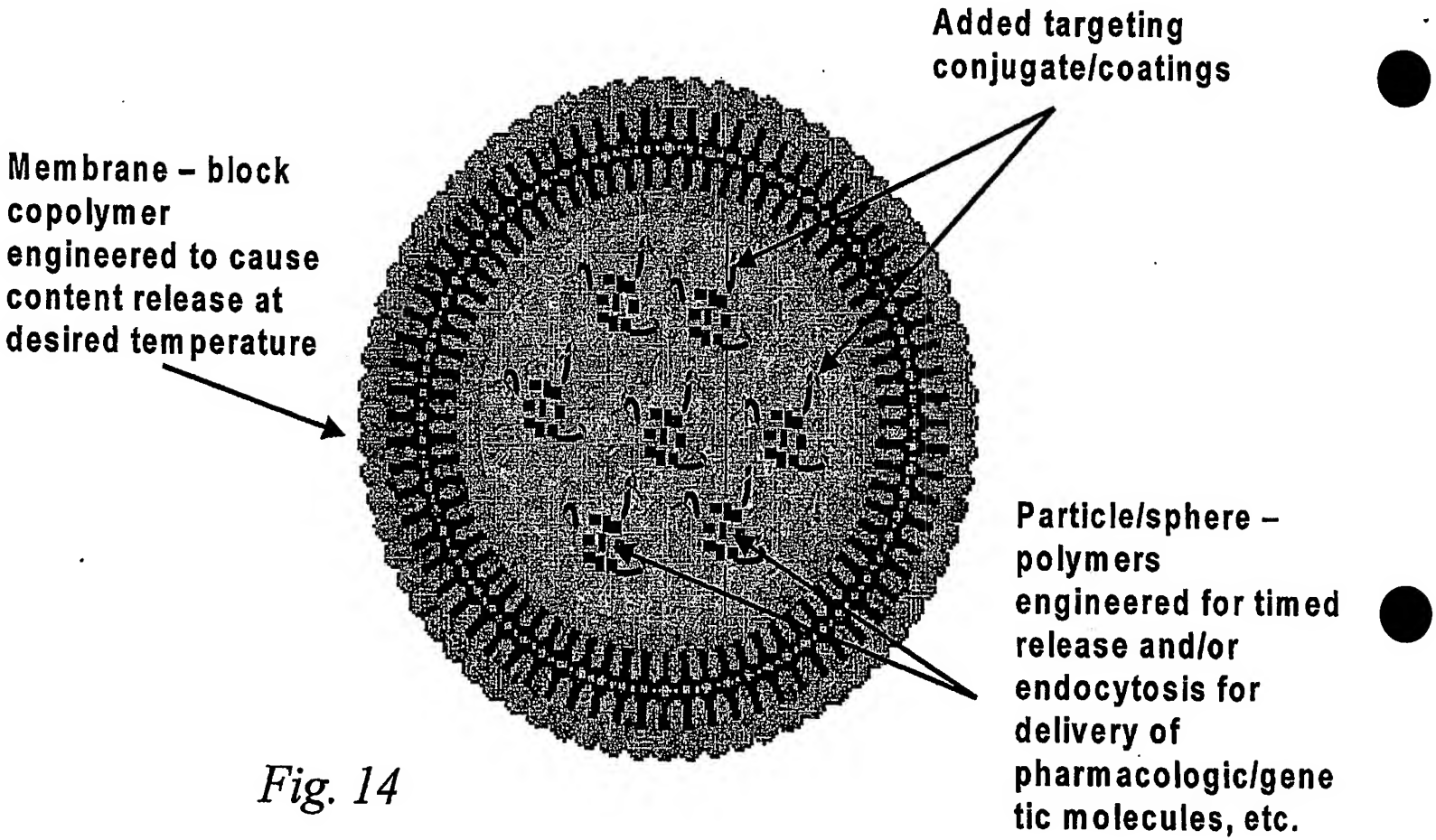
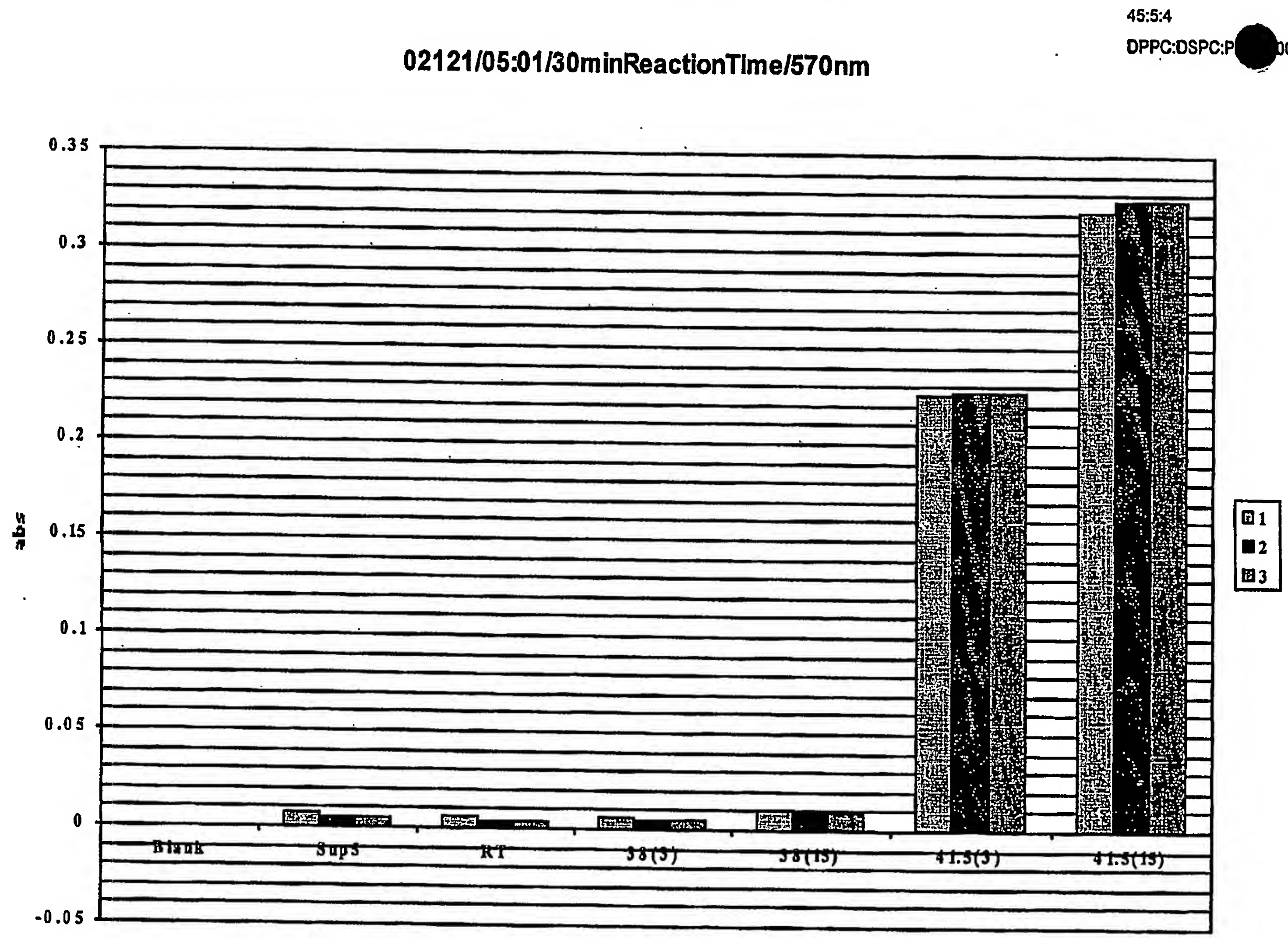

Added targeting conjugate/coatings
Membrane – block copolymer engineered to cause content release at desired temperature
Particle/sphere – polymers engineered for timed release and/or endocytosis for delivery of pharmacologic/genetic molecules, etc.

Fig. 14



45:5:4
DPPC:DSPC:P 00
02121/05:01/30minReactionTime/570nm
abs
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
Blank
Sups
RT
38(3)
38(15)
41.5(3)
41.5(15)
1
2
3

Fig. 15

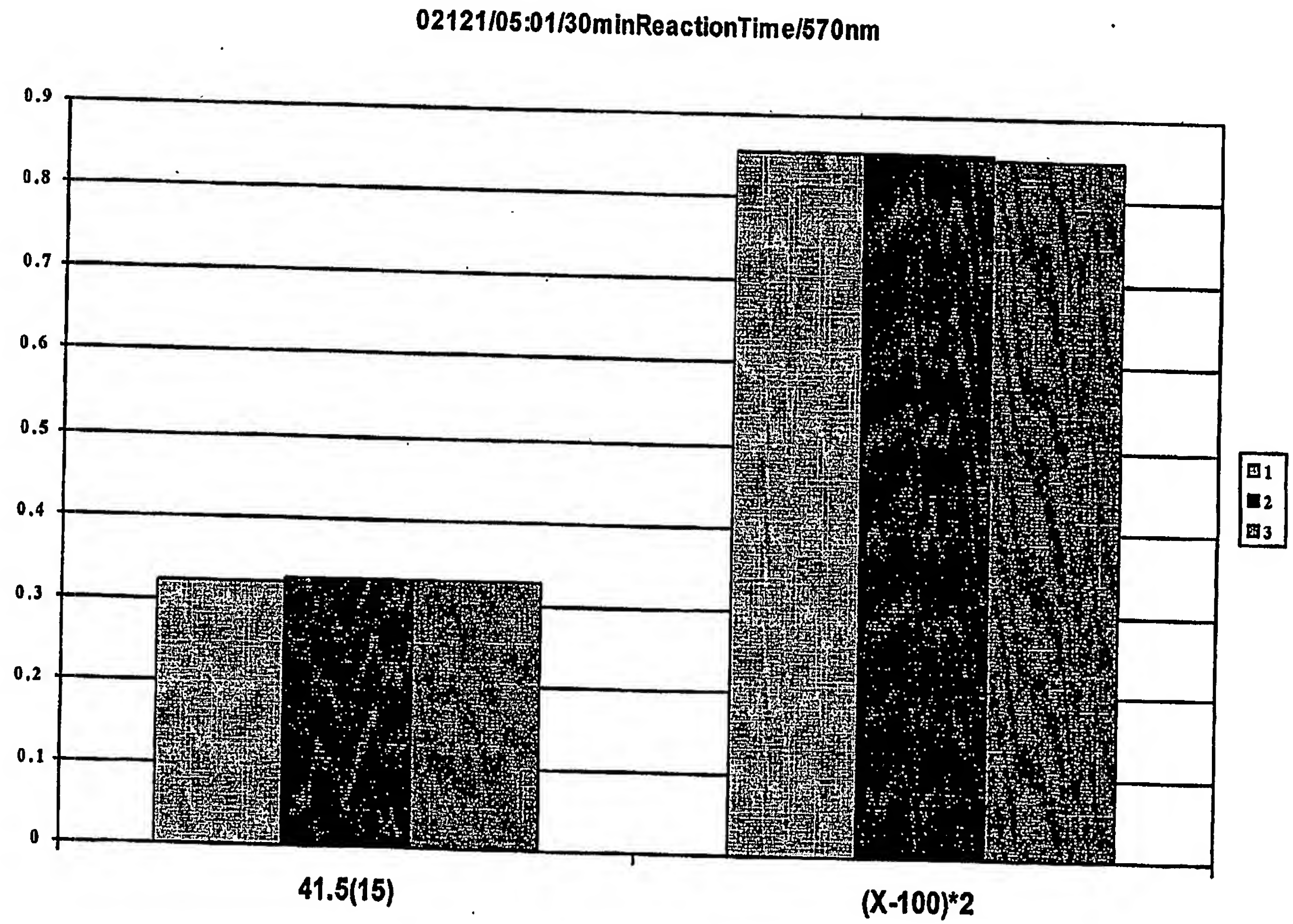


Fig. 16

NOTE: X-100*2 due to dilution upon sample mixture with 10% Triton X-100 during detergent trial

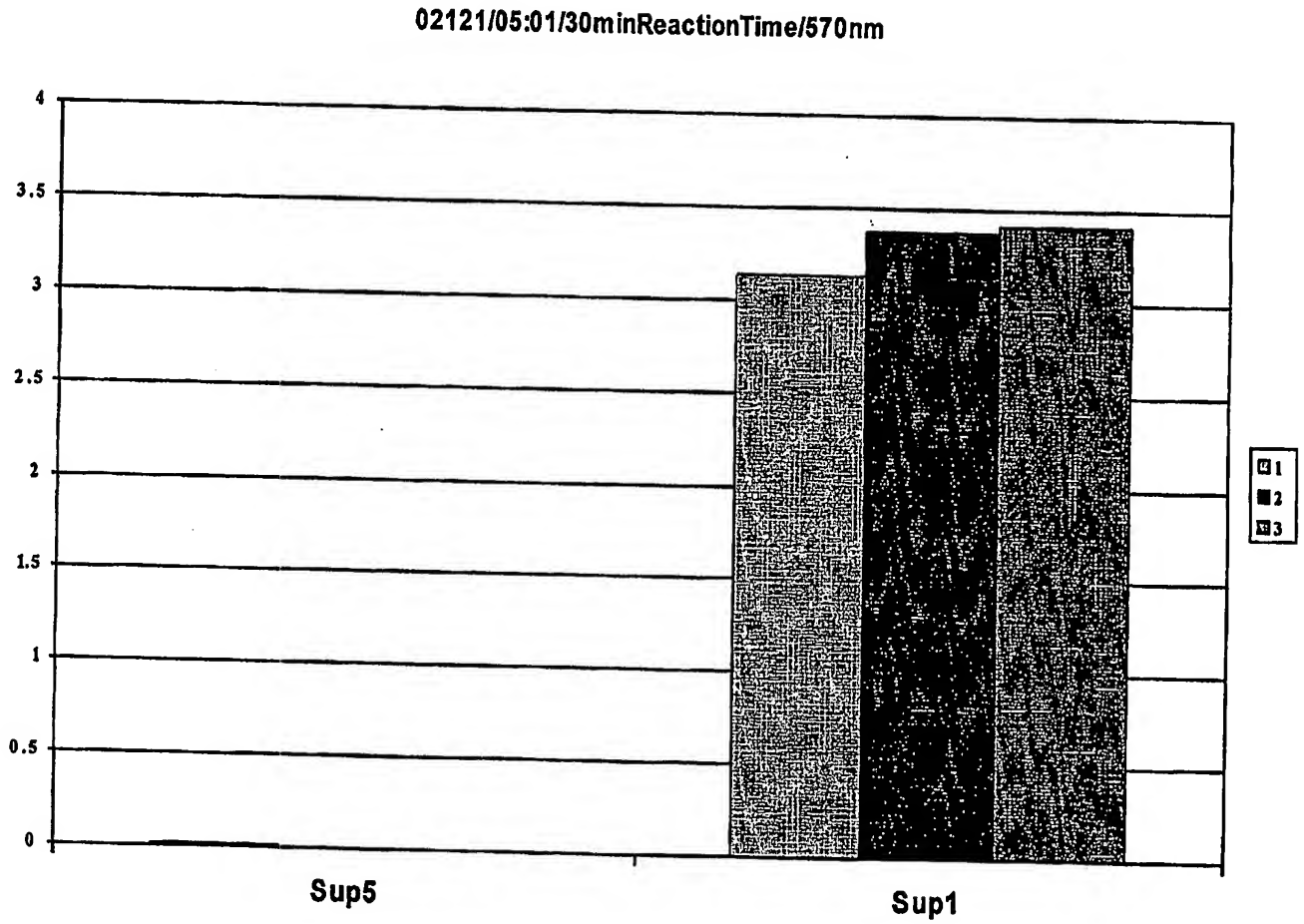


Fig. 17

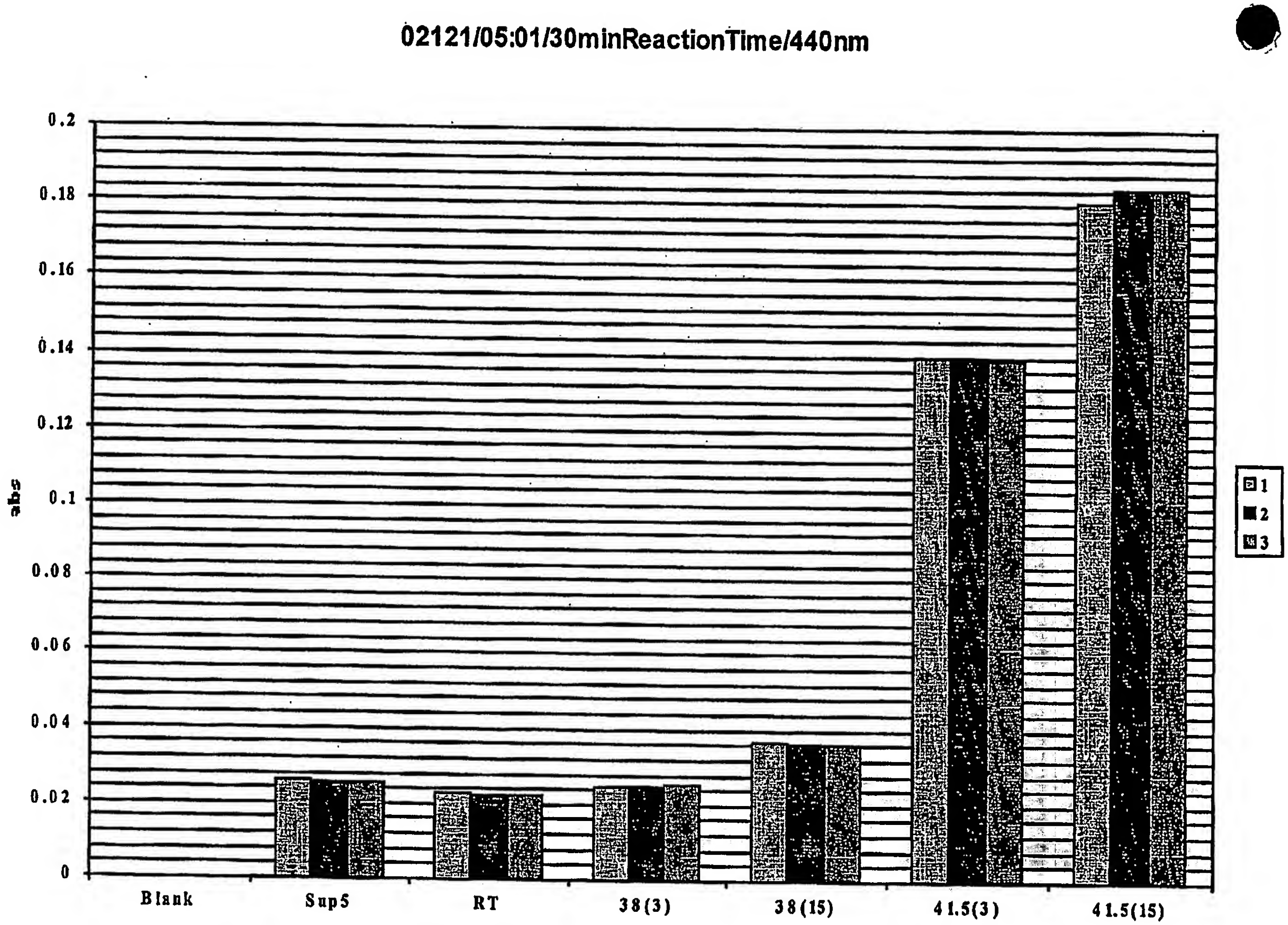

02121/05:01/30minReactionTime/440nm
abs
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Blank
Sup5
RT
38(3)
38(15)
41.5(3)
41.5(15)
1
2
3

Fig. 18

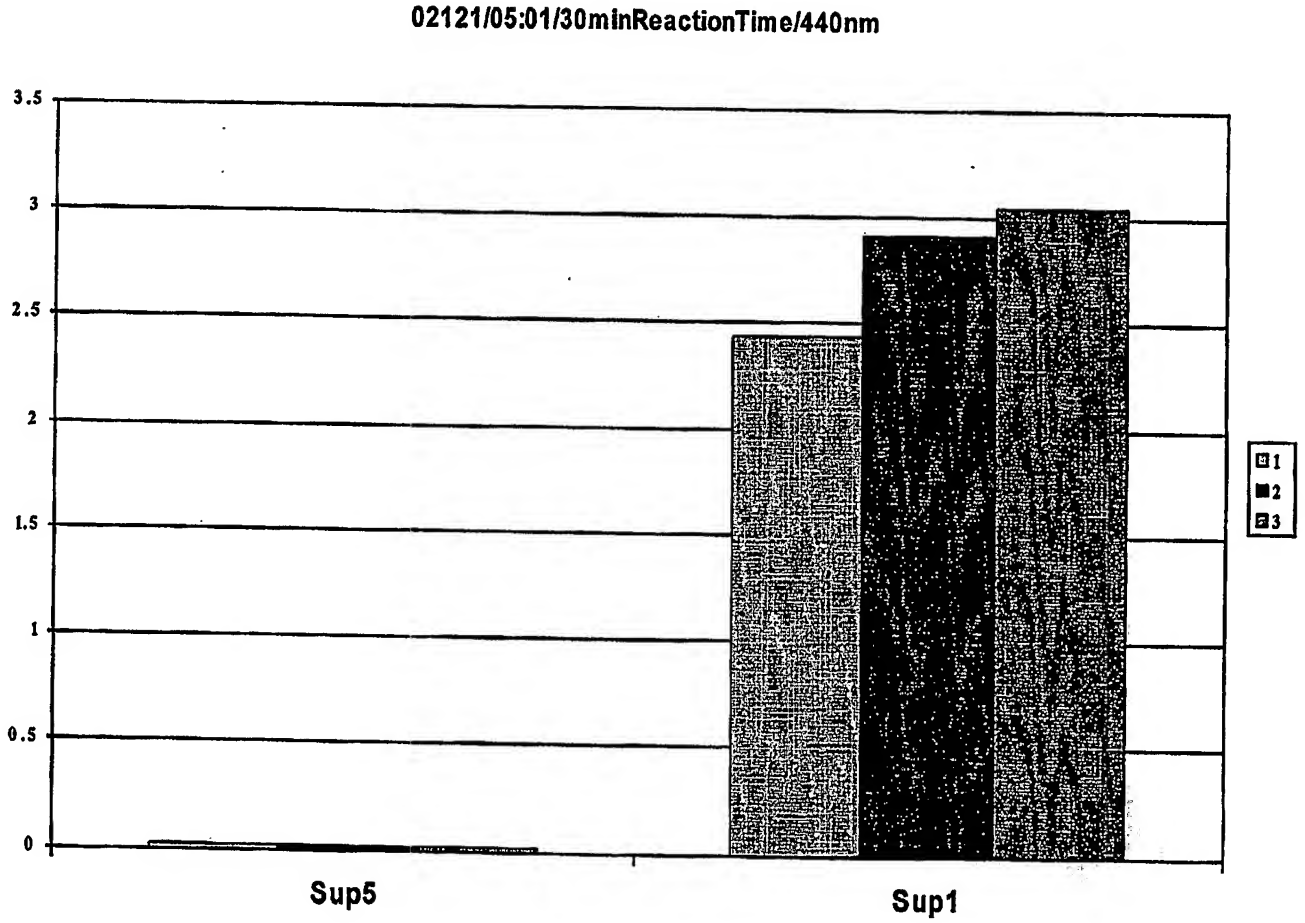

02121/05:01/30minReactionTime/440nm
3.5
3
2.5
2
1.5
1
0.5
0
Sup5
Sup1
1
2
3

Fig. 19

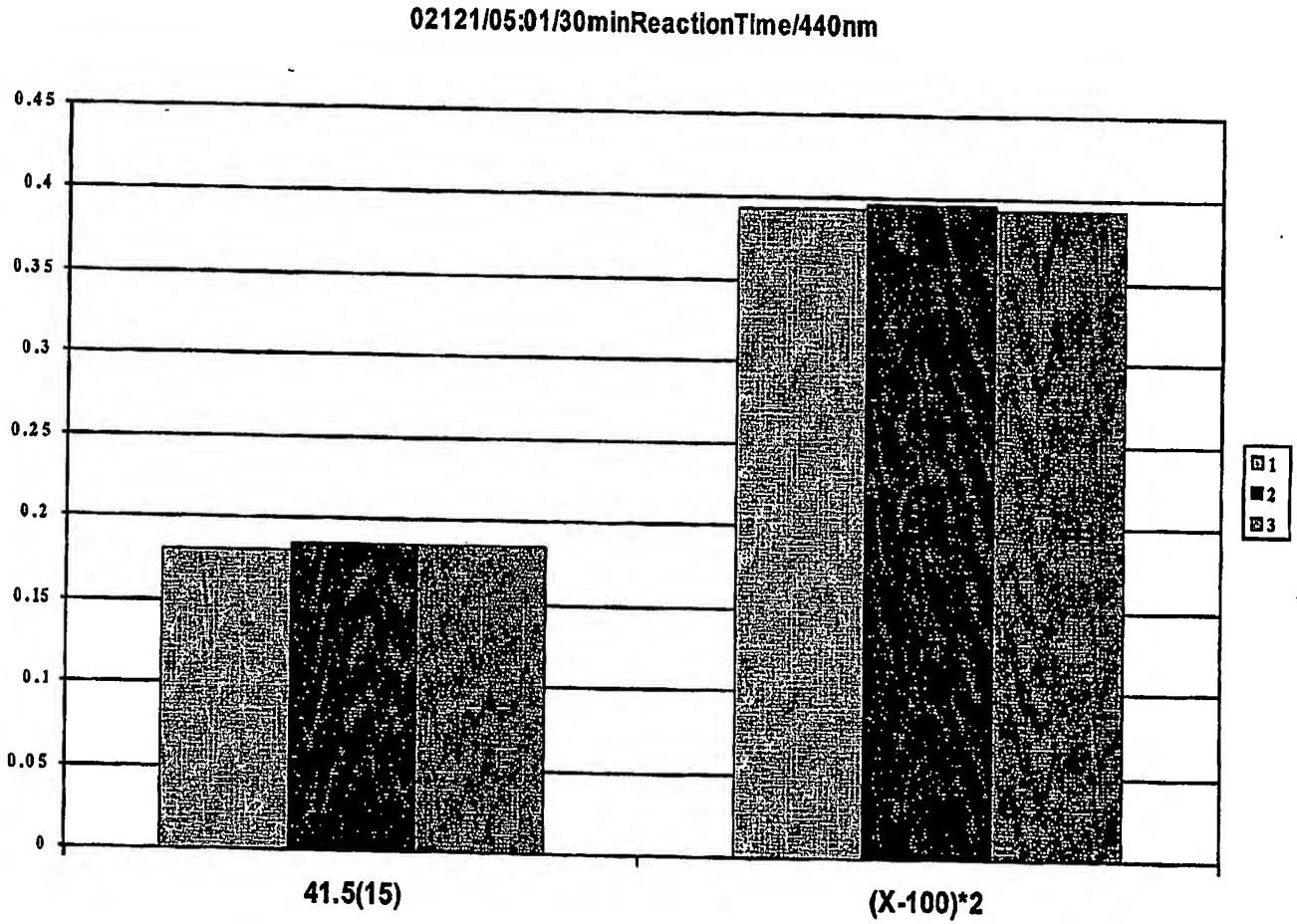

02121/05:01/30minReactionTime/440nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
41.5(15)
(X-100)*2
1
2
3

Fig. 20

DPPC:DSPC
9:1

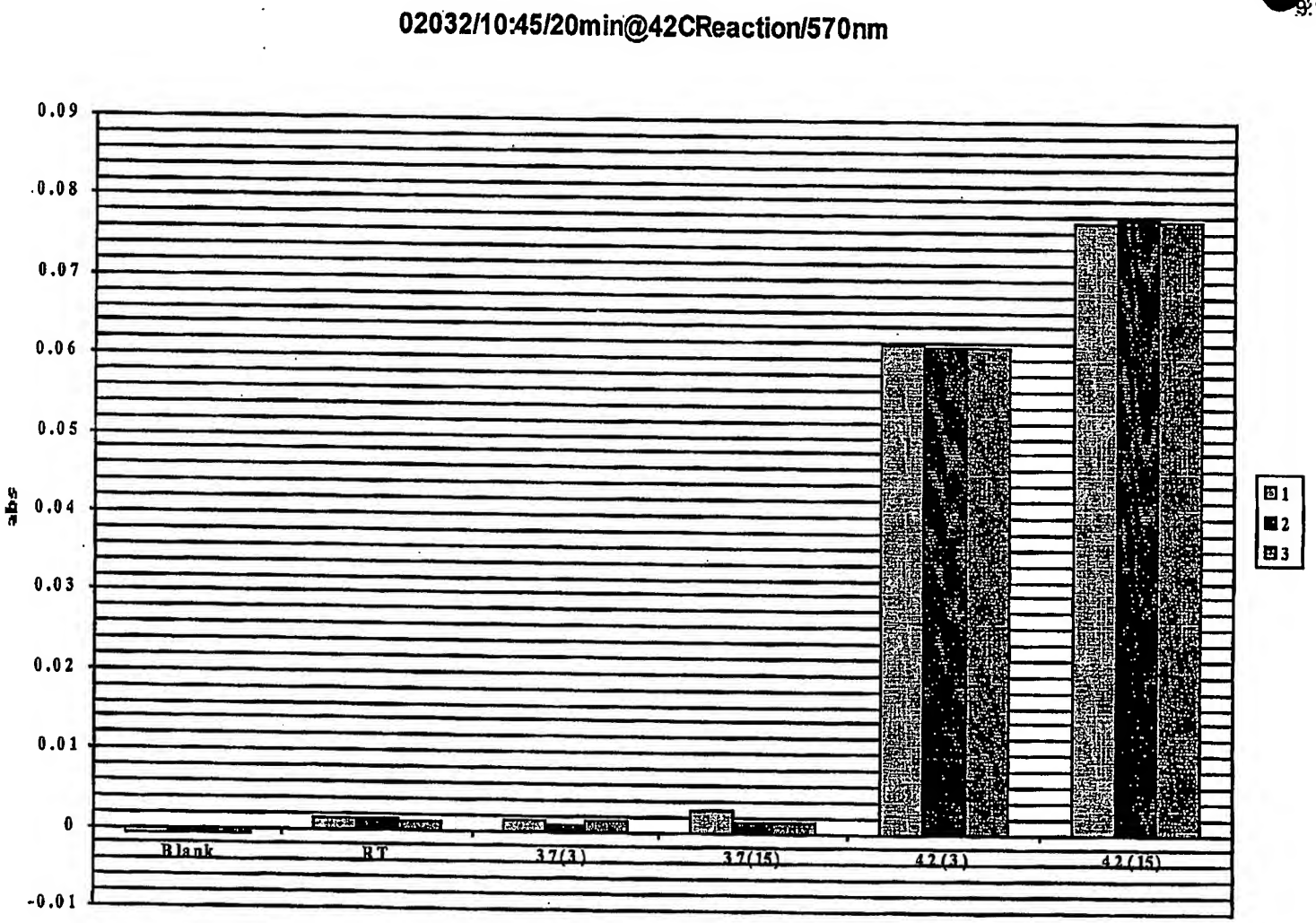


Fig. 21

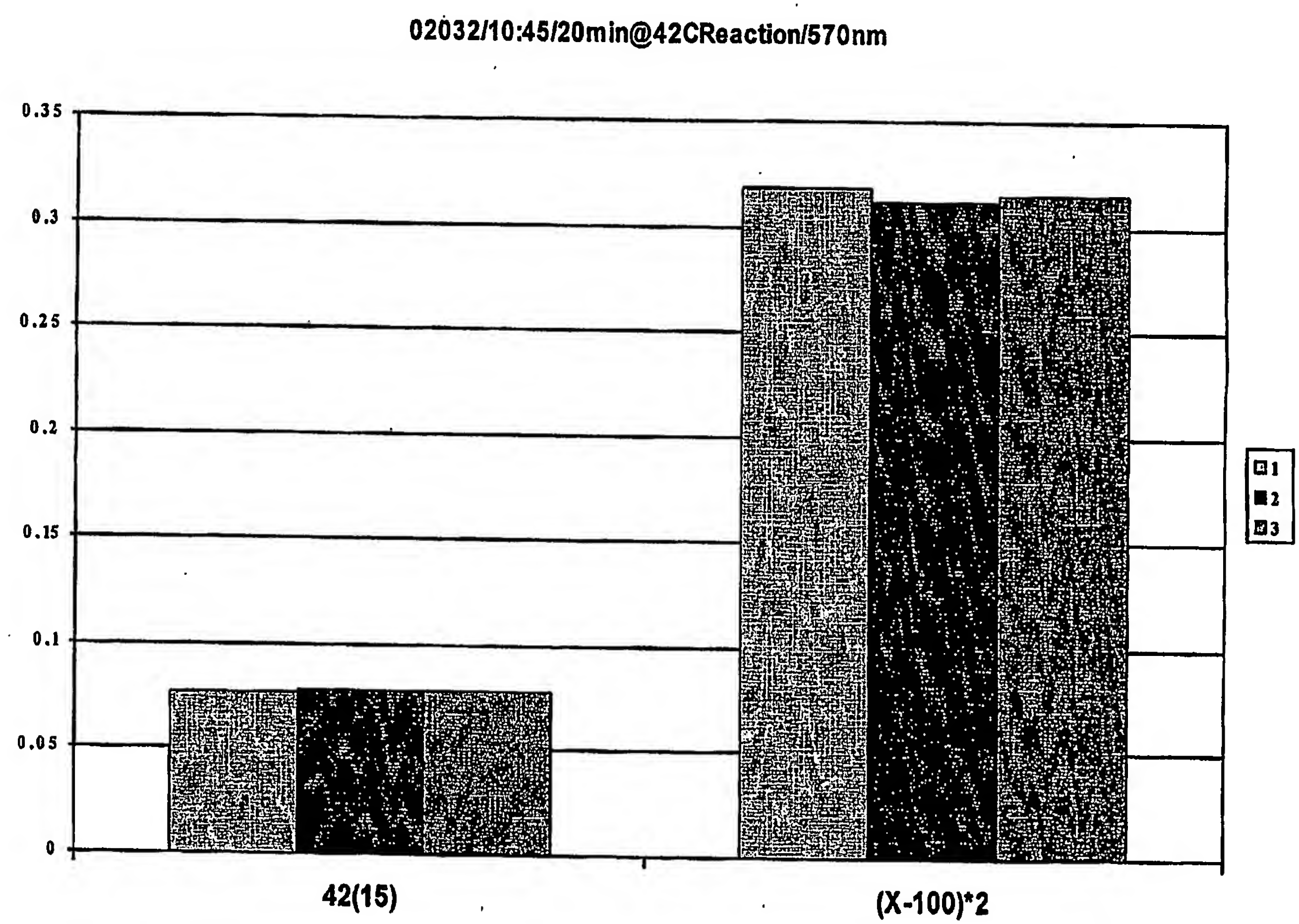


Fig. 22

NOTE: X-100*2 due to dilution upon sample mixture with 10% Triton X-100 during detergent trial

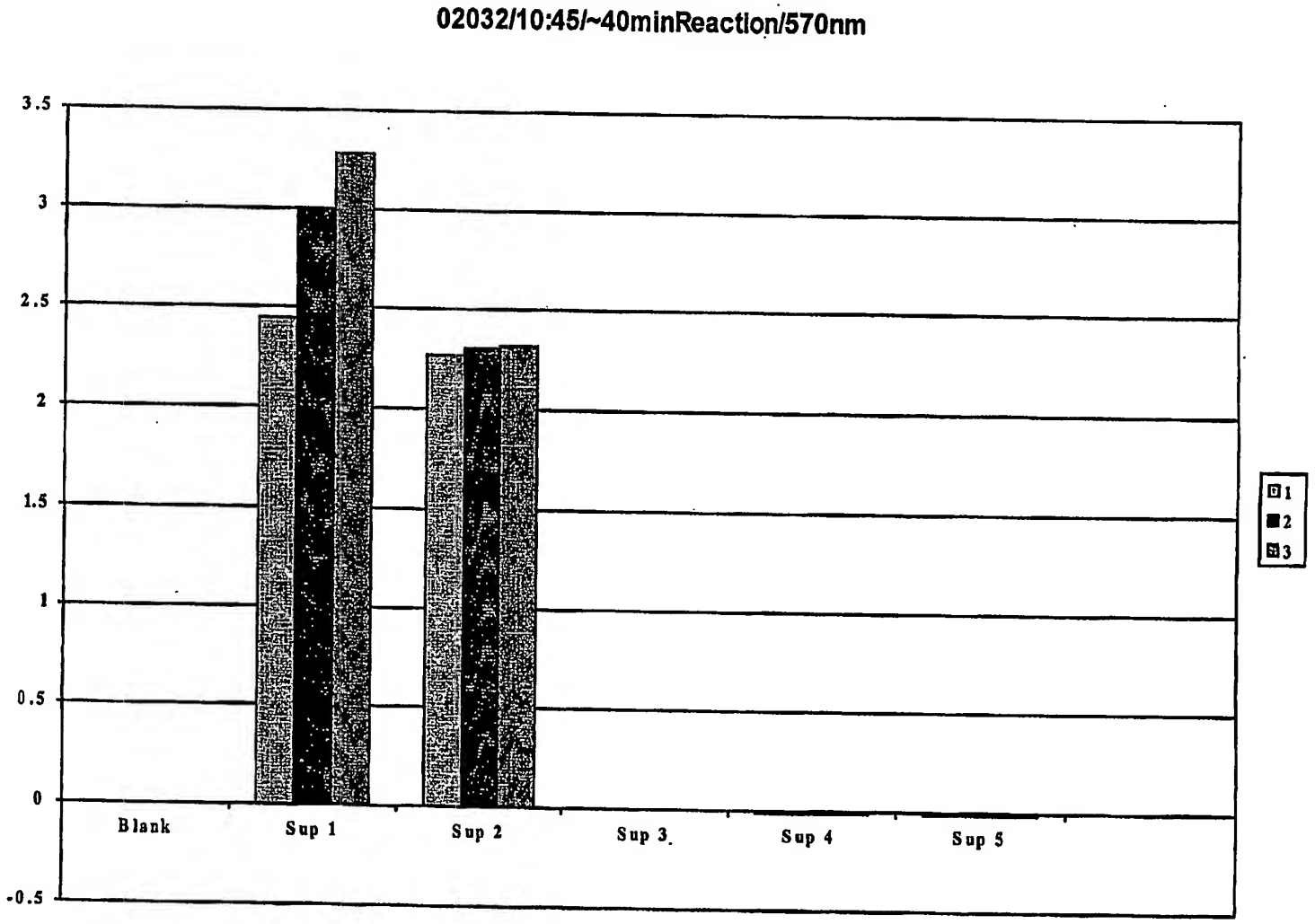

02032/10:45/~40minReaction/570nm
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
Blank
Sup 1
Sup 2
Sup 3
Sup 4
Sup 5
1
2
3

Fig. 23

10:45/20min@42CReaction/440nm

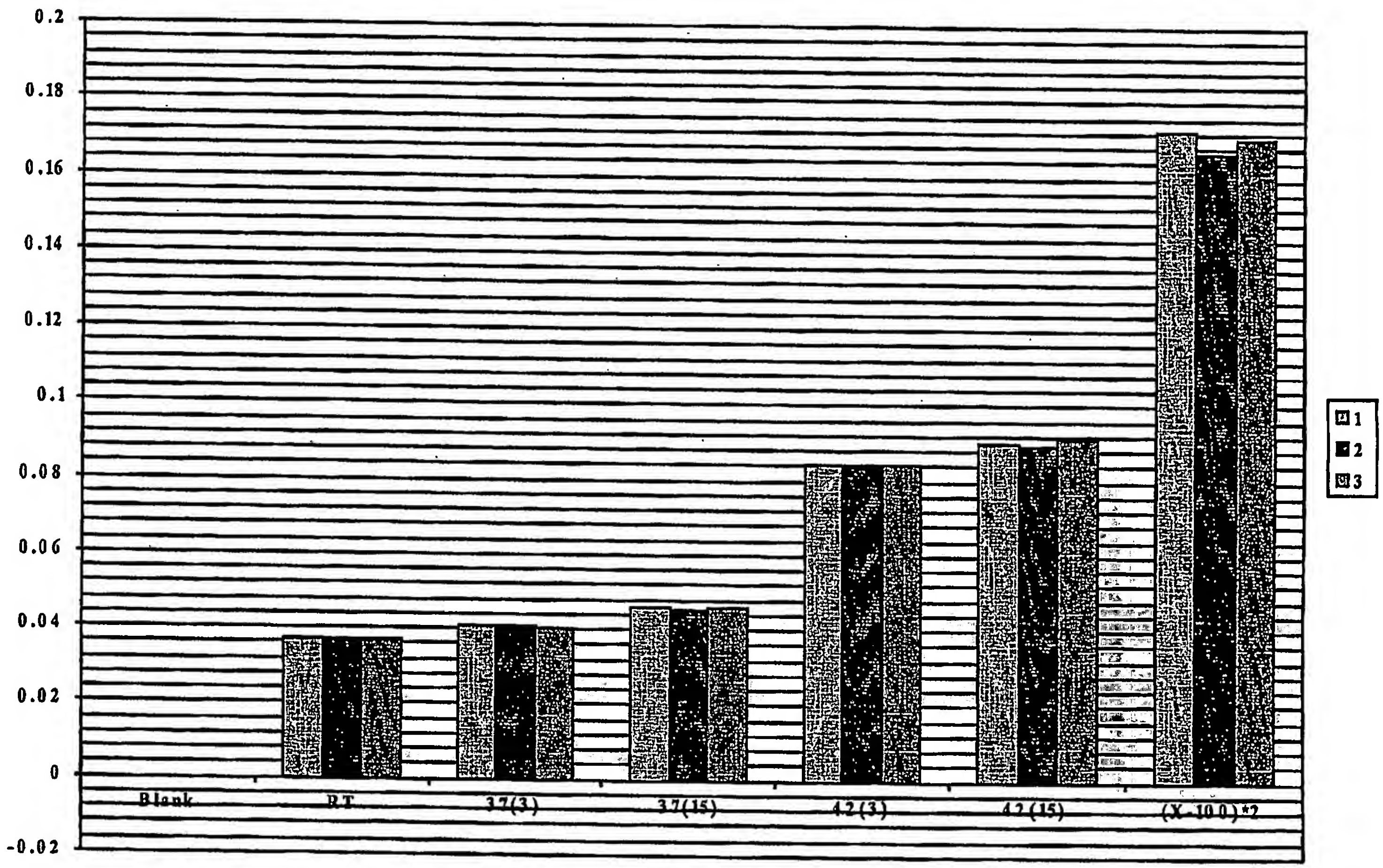


Fig. 24

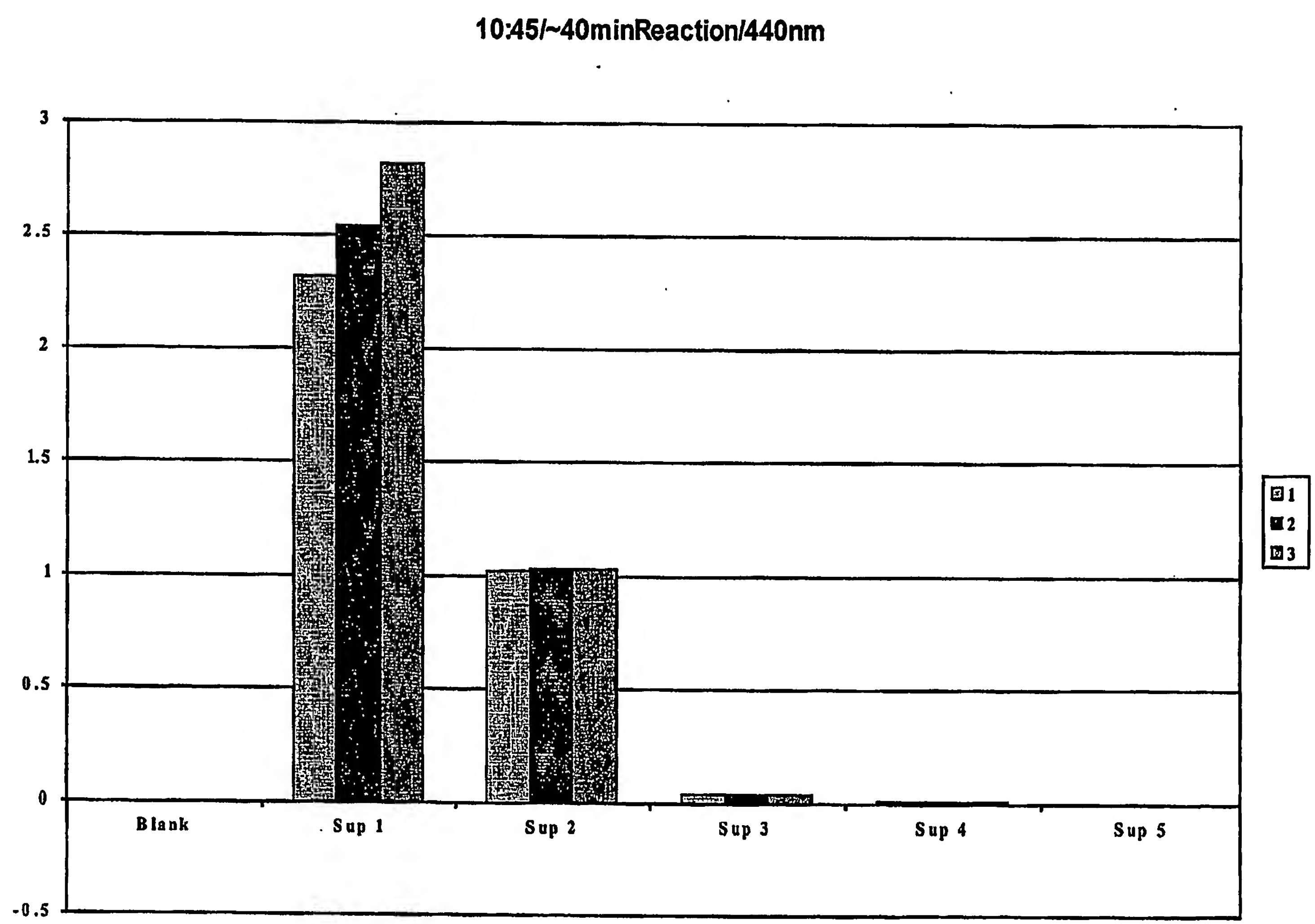

10:45/~40minReaction/440nm
3
2.5
2
1.5
1
0.5
0
-0.5
Blank
Sup 1
Sup 2
Sup 3
Sup 4
Sup 5
1
2
3

Fig. 25